Home Office

Animals (Scientific Procedures) Act 1986

Non-technical summaries for project licences granted July - September 2023

Contents

1. Evaluate the Safety and Performance of Robotically Assisted Medical Devices

Project duration

5 years 0 months

Project purpose

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

Robotic Surgery, Minimal Access Surgery, Advanced Energy, Advanced Imaging, Surgical Instrumentation

Animal types	Life stages
Pigs	adult

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

To support the development of robotically assisted surgical medical devices to enable more patients to get the benefits of minimal access surgery.

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

Why is it important to undertake this work?

Minimal Access Surgery (MAS), otherwise known as keyhole surgery, is carried out by creating small incisions on the patient's abdomen through which surgical instruments and surgical endoscopes can be used to operate on the patient without compromising the safety and efficacy of care as opposed to creating large incisions across the patient's

abdomen i.e. open surgery. MAS has several advantages over open surgery including a reduction in infection, reduction in blood loss, shorter operating times and quicker post operative recovery times. Collectively these benefits can improve clinical outcomes and reduce the length of hospital stay. Although MAS has several advantages over the limitations of open surgery, it is a complex and skilful procedure which can take years for a surgeon to master.

Examples of current limitations to MAS include the reliance on remote vision which gives the operating surgeon a loss of freedom, highly skilful hand-eye coordination is required as well as stability as movements with instruments are counterintuitive and tends to have poor ergonomic outcomes leading to long term pain, discomfort and debilitation for the surgeon.

More recently, Robotic surgery is a growing area which has the added benefit of assisting surgeons to perform technically challenging MAS surgery allowing them to operate with greater control and stability of instruments, improved ergonomic comfort and visual feedback.

By developing robotic technologies through instrumentation, visualisation, hardware and software, this work will help improve patient outcomes, reduce complications and increase the number of patients that can benefit from the advantages of minimal access surgery by developing advanced technology to be used robotically.

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

Safety and performance studies will provide data towards the design and development of new medical device products to improve patient outcomes and reduce the number of surgical complications by using technology that can improve surgical flow, precision, mobility, and ergonomics.

In addition to this project resulting in the development of new medical devices, it may also lead to patent applications, scientific publications and conference presentations which will be used to disseminate key scientific findings.

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

The outputs of this project will have social, political and environmental benefits. Patients will benefit from the products developed in this project as the introduction of new robotic medical devices will allow more patients to gain access from the benefits of Robotics and Minimal Access Surgery (MAS) such as reduced blood loss, reduced risk of infection, improved instrument stability and vision of surgical field and improved ergonomics for the operating surgeon. In summary, the benefits are as follows:

Social: Patients will benefit from having access to advanced technologies enabling more surgeons to carry out Minimal Access Surgery via robotics. This will improve patient outcomes.

Environmental: Increasing the number of patients who have access to MAS will reduce the cost burden on health care institutions by reducing hospital stay.

Political: Increasing the number of patients that have access to robotic MAS procedures align with government goals of reducing waiting lists while advancing the standard of care.

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

Output will be maximised as it will feed into the design and development cycles of future products.

Dissemination of new knowledge through publications, presentations and evidence used towards regulatory submissions.

Where confidentiality agreements allow, we will publish the information via peer- reviewed scientific journals and conference presentations in addition to patent applications.

One of the key goals will be the dissemination of results in seminars, conferences, and peer-reviewed articles with open access, in order to promote the general advancement of the fields studied. Negative findings may be published to avoid duplication of work by other groups.

Species and numbers of animals expected to be used

- Pigs: 190

Predicted harms

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

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

The anatomy and physiology of the porcine model is representative of much of the critical organs, structures, and abdominal geometry within the human body. A porcine model is therefore commonly used as a representative test system for medical devices. Furthermore, the pertinent parameters relative to system and instrument performance evaluation (i.e. the relative workspace, organ size and properties of the live tissue), are representative of that of a human patient.

In-vivo demonstration of the ability of the device to interact with and manage live tissue is crucial for the assessment of additional modern technology.

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

Animals will be acclimatised to the facility. All procedures carried out on animals will be done under general anaesthesia, (following withdrawal of food only for up to 24 hours) and by experienced surgeons. For proof of concept testing, specific surgical tasks and/or procedures will be carried out. For non-recovery studies, the animal will then be terminated following testing. Post mortem samples may be collected on areas where for example energy was delivered and/or surgical tasks were carried out.

For recovery studies, a single procedure, which may include additional minimally invasive surgical tasks such as vessel sealing and cutting will be carried out, and typically the animal recovered for a maximum of 35 days. The welfare of the animal will be of priority with specific end-points identified, and the animal will be euthanised if it reaches or is likely to reach the identified end-points.

To maximise animal usage, multiple aspects and/ or devices will be tested in a single session for proof of concept testing in non-recovery animals wherever scientifically possible.

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

A proportion of animals used in these studies will not experience any adverse effects as the studies will be conducted under terminal anaesthesia. 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. We would expect the animals to show full recovery within 7 days of surgery. If this is not the case the animals will be humanely killed.

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

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

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

55% of animals will be non-recovery studies and the remaining 45%classified as moderate severity.

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

- Killed

Replacement

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

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

The anatomy and physiology of the porcine model is representative of much of the critical organs, structures, and abdominal geometry within the human body. A porcine model is therefore commonly used as a representative test system for medical devices. Whilst the pertinent parameters relative to system and instrument performance evaluation i.e., the

relative workspace, organ size and properties of the live tissue, are representative of that of a human patient.

In-vivo demonstration of the ability of the device to interact with and manage live tissue is crucial for the assessment of additional modern technology.

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

As part of the product development plan, all devices would have been tested prior to the study using synthetic and tissue bench top models, and/or cadaveric tissue.

This bench top testing will ensure that only the most clinically representative products will be tested in vivo.

Why were they not suitable?

In-vivo demonstration of the ability of the device to interact with and manage live tissue is crucial for the assessment of additional modern technology.

Although in vitro experimentation will be conducted, this alone cannot provide all the necessary conditions to assess the safety and performance of medical devices before going in human.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 proof of concept studies will use the minimum number of animals per device to be able to answer our scientific questions. Multiple procedures and/ or tasks will be conducted in a single animal where appropriate. The studies intended for GLP regulatory purposes will be done in accordance with regulatory guidelines (e.g. FDA 3-9 animals per group/ time point).

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

All experimental designs will consider the 3R's to reduce, replace and refine experimental design. For proof of concept studies/ early feasibility/ pilot studies, power calculations are not appropriate and sample sizes will be calculated based on operational capacity and constraints which is in line with ARRIVE 2 guidelines. In general, proof of concept studies/ early feasibility/ pilot studies, a single (non- recovery) animal will be used multiple times if it can be done so safely and scientifically to reduce the number of animals required. For studies required for regulatory submission in order to establish device safety, the regulatory guidelines will be followed.

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

All devices will have been tested on the bench before hand and any data that can be generated without the use of an animal will be done so. For proof of concept studies, a single (non-recovery) animal will be used multiple times if it can be done so safely and scientifically to reduce the number of animals required.

All tissue which can be shared will be shared.

Refinement

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

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

Pigs will typically be used for all experiments because they are the most appropriate species to determine performance and safety with respect to the devices tested. Their neuroanatomy and physiology are very similar to that in humans.

Recovery studies will only be performed once the surgical technique has been defined as much as possible using cadavers and/or terminally anaesthetised animals.

All methods used for recovery animals will be refined to minimise any pain; these include appropriate provision of analgesia, use of local anaesthetics where possible and close monitoring of animals by large animal veterinarians and advanced trained animal technicians to recognise any adverse effects. All animals will be trained and habituated to the environment, staff and handling techniques prior to study to minimise stress.

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

Porcine models are the most representative of human physiology and anatomy, therefore in order to test the safety and performance of these devices before going into human.

Porcine models are required for these medical devices and smaller models pose physical restraints in terms of operating volumes and organ sizes. It should be noted that the majority (50%+) of studies will be conducted on terminally anaesthetised animals.

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

To minimise discomfort/harm to the animals where possible studies are non-recovery in terminally anaesthetised animals with defined humane endpoints.

All animals will receive appropriate peri-operative care in terms of anaesthesia and pain management both during and after a surgical intervention.

Our in-house large animal vets’ expertise further enhances animal welfare by providing close collaboration with dedicated animal care staff and ready access to highly skilled advice. Specific recovery plans have been designed to ensure the best recovery of any animal post-procedure and involve high levels of monitoring.

All animals are habituated to the environment and all recovery animals are trained prior to use for all handling procedures, such as use of a restraining crate.

Least invasive route of substance administration, appropriate needle gauge and local anaesthesia will be used where possible. Negative control groups (baseline groups) will be minimised whenever statistically feasible.

All individual study plans are reviewed by the Study Manager, PPL Holder, Named Veterinary Surgeon and key study staff including consideration of justification and implementation of refinement and reduction as part of the local protocol review process.

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

We will follow the NC3Rs guidelines on the "Responsibility in the use of animals in bioscience research" and consult all the relevant references listed therein, e.g. NC3Rs Analgesia. We will also follow the LASA Principles for preparing for and undertaking aseptic techniques and follow the PREPARE and ARRIVE 2 guidelines.

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

Monitoring publications and the NC3Rs website for new and alternative models that could be implemented as part of this project, or for review purposes prior to starting new models. In addition, articles on advances in the 3Rs are regularly published on the internal BSU Users News Forum and other relevant information is circulated by AWERB. Whenever possible we will implement these refinements into our studies.

2. Induction, evaluation and prevention of intra-mammary infections in ruminant livestock

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

Mastitis, Ruminants, Genetics, Immune mechanisms, Mammary infection

Animal types	Life stages
Sheep	adult, juvenile, neonate, pregnant
Cattle	adult, pregnant

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

We aim to improve mammary health and reduce the dependence on antibiotics through a combination of population genetics, testing of novel antimicrobial extracts and manipulation of immune responses within the mammary gland of ruminant 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?

Intra-mammary infections causing mastitis represent one of the most important production-associated diseases in ruminants worldwide. The most common bacterial species

associated with dairy cow and sheep mastitis are Staphylococcus aureus, Streptococcus uberis, Escherichia coli, Mannheimia haemolytica and several species of coagulase-negative staphylococci. Clinical mastitis is an important animal welfare issue and can lead to death, premature culling and reductions in milk quantity and quality. Mastitis treatment is dependent of the use of antimicrobials at various stages of the lactation cycle. This work will help to reduce the dependence on antimicrobials through a combination of host genetics, testing of alternatives to antibiotics and furthering our understanding of innate and adaptive immune mechanisms within the mammary gland which may be exploited in the development of future disease control strategies.

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

Novel information on host-pathogen interactions and mechanisms of immunity within the mammary gland.

Novel longitudinal data on the impact of sheep mastitis and the associated pathogens.

Identification of regions of the sheep genome associated with resistance to infection, the response to infection or resilience in the face of infection.

An archive of bacterial isolates from clinical and subclinical infections which will underpin future funding applications.

Potential products include novel alternatives to antimicrobial use in dairy cattle.

A range of peer-reviewed publications, submissions to public access data bases and knowledge exchange activities to stakeholders.

Novel animal models for future study of mechanisms and intervention strategies infections.

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

Ruminant livestock: Clinical mastitis is a painful condition that can severely impact the health and welfare of affected animals. Prevention of intramammary infections through vaccination and effective treatments will alleviate the suffering caused by intramammary infections.

Farmers and primary producers: Huge direct and indirect production losses are associated with intramammary infections in all ruminant livestock species and livestock production systems worldwide. Disease prevention or effective treatment of infected animals will increase the efficiency of livestock production systems, reduce wastage, and contribute to mitigating the effects of greenhouse gas emissions.

Research scientists: The data generated from this research will interest researchers seeking to improve our understanding of the response of the mammary gland to an intramammary infection. Publication of the data generated throughout the course of this project will contribute to scientific knowledge and the development of future collaborations and funding applications.

General public: Public awareness of farm animal welfare is increasingly driving improvements in animal husbandry. Efforts to improve dairy cow health and welfare are no exception.

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

The outputs of this work will be maximized by collaborations with other investigators across the UK and internationally. New data will be presented at farmer orientated events. New knowledge resulting from this research will be published in peer reviewed journals, newsletters and presented at national and international meetings.

Unsuccessful approaches will be documented alongside successful approaches in the resulting publications.

Species and numbers of animals expected to be used

- Cattle: 30
- Sheep: 400

Predicted harms

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

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

Mastitis in livestock production is common in lactating sheep and dairy cows. These species are the natural hosts of the bacterial infections we propose to study, and we have experience of challenging both species to induce disease under controlled laboratory conditions. Studying disease in its natural host is preferable to model organisms where the disease may not fully represent the natural condition. When studying disease interventions such as vaccines or antimicrobials, it is preferable to test these in the target host species.

Life stages: As this project focuses on lactating dairy ruminants, it will use female sheep and dairy cattle from their first lactation towards the end of their productive life. However, for pedigree and genetics, we may be required to take a limited number of blood samples from breeding males, neonates and juvenile individuals.

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

Sheep: Depending on the type of study, either annual blood and milk sampling procedures carried out on a sheep flock of over five years with animals returned to the breeding flock following sampling or challenge studies which require daily milk and blood samples over a period of 3-5 weeks.

Sampling and inoculation procedures:

Blood samples for the preparation of DNA, RNA, leukocytes, and serum.

Hand or automatic milking to provide milk samples for somatic cell counting and quantitative and qualitative bacteriology and to maintain lactation following removal of the lamb.

Injection of antigens at a range of sites to stimulate peripheral immunity.

Intra-mammary infusion of antigenic, antimicrobial, or immunomodulatory substances. Intra-mammary inoculation of bacterial cultures to induce an intra-mammary infection.

Cattle: All cattle studies will employ the state-of-the-art dairy facilities on site. All lactating dairy cows will be purchased from commercial farms.

Sampling and inoculation procedures:

Blood samples for the preparation of DNA, RNA, leukocytes, and serum.

Automatic milking to provide samples for somatic cell counting, quantitative and qualitative bacteriology and to maintain lactation.

Injection of antigens at a range of sites to stimulate peripheral immunity. Intra-mammary infusion of antigenic or antimicrobial substances.

Intra-mammary inoculation of bacterial cultures to induce mastitis.

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

Sheep: For longitudinal field studies of up to five years the expected impact and adverse effects will be minimal and associated with annual blood collections for serum, cells, and nucleic acid isolation.

Samples of milk collected from healthy lactating ewes for SCC and bacteriology are not expected to adversely impact the animals. Mammary secretions from sheep that develop clinical mastitis over the course of these studies will be sampled for diagnostic bacteriology prior to antimicrobial treatment according to veterinary advice.

Sheep and dairy cattle: For laboratory challenge studies in sheep or dairy cattle, animals will be selected based on low somatic cell count and absence of an intra-mammary infection. Animals will be grouped and held in purpose-built animal accommodation on site, monitored by the PPL holder and trained animal care technicians and identified as healthy by the NVS prior to enrolling in the study.

Animals may be inoculated at a range of locations including intra-mammary infusion with immuno- stimulatory molecules, antimicrobials or vaccine formulations. Systemic inoculation is not expected to have adverse impacts at injection sites above mild inflammation and in some cases localised granuloma formation. Intra-mammary infusion may induce a mild to moderate inflammatory reaction which is expected to be transient in nature with limited adverse effects. All infusions will be pre-tested for cellular toxicity in vitro prior to inoculation in vivo. Pilot studies using small numbers of animals will be carried out prior to larger studies. Animals will be challenged by intra-mammary infusion of bacteria at predetermined volumes and concentrations to induce intra-mammary infections. Challenge is not expected to exceed moderate severity and all animals will be monitored twice daily. Sampling will include milk samples for bacteriology, cellular and cytokine analysis and blood samples for serum, antigen re-stimulation and cytokine analysis. These studies will generally last between 4 and 8 weeks and are not expected to exceed moderate severity. Any adverse reactions will be treated after veterinary consultation. Depending on the type of study, sheep or dairy cows may be euthanised for collection of mammary or other tissues at post-mortem or treated and returned to the flock or reused in other studies according to cumulative harm guidelines.

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

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

Sheep longitudinal field studies expected level of severity associated with bleeding and milk sampling: Mild and subthreshold respectively 100%.

Sheep challenge studies: Between 50 and 70% of animals are expected to experience moderate severity with the rest experience mild severity.

Dairy cow challenge studies: Between 50 and 70% of animals are expected to experience moderate severity with the rest experience mild severity.

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 investigating diseases that impact ruminant livestock health and welfare and developing intervention strategies to mitigate the effects of these diseases. Studying disease pathogenesis and the innate and adaptive cellular immune responses to infection in its natural animal host is essential for this process as in vitro alternatives are not available.

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

In vitro methods and non-animal alternatives including primary and transformed mammary epithelial cell lines and mammary organoid cultures will be used whenever possible. In vitro cellular toxicity assays will be used to assess the safety of immune modulators and alternative antimicrobial formulations prior to in vivo studies.

Why were they not suitable?

It is not possible to quantify systemic responses, responses targeted to the mammary gland or define the complexity of immune responses solely using non-animal-based systems or organ cultures. Non- animal derived alternatives are not suitable to study the development of disease, the response of an animal over time or to determine if a vaccine or antimicrobial provides protection These can only be carried out in properly controlled studies using live animals.

Reduction

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

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

The sheep numbers are based on the number of breeding ewes purchased to provide lambs over the next five years. Each of these ewes will be blood sampled annually and milk samples will be collected at lambing. The rams used for breeding will also be blood sampled to provide DNA and RNA for genotyping and pedigree studies. Sheep will also be used for intra-mammary challenge studies to investigate immune mechanisms and testing vaccines, antimicrobials and immune modulatory compounds.

Dairy cow numbers are based on the estimated number of animals required to test plant-based alternatives to antimicrobial treatment for mastitis in a recently funded EU-Horizon 2020 grant. Additional animals will be used in to test vaccines, immune modulators and further model development over the five years of the licence.

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

All animal studies are planned in consultation with bio-statisticians to ensure adequately powered group sizes for the most statistically robust analyses while minimising the number of experimental animals, prior to consideration by the local AWERB. Consideration of the 3R's is a key component of the experimental design and evaluation process. When testing novel formulations, pilot studies will be carried out prior to provide data for power analysis of group sizes and to ensure that animal welfare is not compromised.

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

When testing novel formulations pilot studies will be carried out to provide data for power analysis of group sizes to ensure that animal welfare is not compromised.

Refinement

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

Which animal models and methods 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 longitudinal sheep study no animal model is required. This study will investigate naturally occurring intra-mammary infections (IMI) in a sheep flock over a five-year period from first to their fifth lactation. Licenced procedures will include blood sampling to prepare serum and white blood cells for DNA and RNA. This is a mild procedure where volumes will not exceed local guidelines. No adverse effects of blood sampling are envisaged. Blood will be taken by superficial venepuncture on a maximum of two occasions in any 12-month period. There may be mild discomfort at the time of blood sampling, but this should be transient and should not require treatment with analgesic drugs.

From each healthy ewe during lactation, 5-10 ml milk samples will be collected from each mammary half for bacteriology and SCC. This is a sub-threshold, non-invasive procedure that will not cause distress or lasting harm to the ewe. For ewes experiencing clinical signs

of mastitis as in current farm practice, samples will be collected for diagnostic purposes under the Veterinary Surgeons Act.

Sheep challenge models: Intra-mammary challenge models have been developed for the three common bacterial pathogens associated with IMI in sheep. These are used to test candidate vaccines, antimicrobial compounds or immune modulators which may be delivered intra-mammary via the teat canal or via a parenteral route and are considered mild procedures. The challenge models include the Gram-negative bacteria, Mannheimia haemolytica and the Gram-positive bacteria, Streptococcus uberis and Staphylococcus aureus. These models induce mild to moderate clinical disease which generally resolve within 7-10 days following challenge. A clinical scoring systems has been developed for each challenge to closely monitor the severity of the infections. This and frequent monitoring ensure that no animal exceeds the severity limit. Sample collection from each animal model includes mammary secretions for bacteriology and SCC. In a disease-free animal, mammary sampling will be sub-threshold but this may increase to mild when an inflamed mammary gland is sampled. Venous blood will also be sampled for serum and immune cells.

Cattle challenge models: Intra-mammary challenge models have been developed for Streptococcus uberis and Staphylococcus aureus. These models induce mild to moderate clinical disease which generally resolves 7-10 days following challenge. A clinical scoring system has been developed for each challenge to closely monitor the severity of the infection following challenge. This and frequent monitoring ensures that no animal exceeds the severity limits.

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

These studies require lactating mammals.

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

All animals under study will be monitored by experienced animal care technicians and the licence holders (PPL; PIL). All studies will include a decision-making plan to deal with clinical signs and to ensure that severity limits are not exceeded. Any adverse effects of treatment or challenge will be assessed by the NVS and appropriate treatment provided or end of study decisions made. Pain management will be provided if required. Animal numbers will be continually assessed and group sizes reduced wherever possible.

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

All studies on site are required to follow best practice and to be authorised by the local AWERB. This committee consists of experts in the fields of animal health and welfare, experimental design and statistical analysis. Published guidance is provided by NC3R (https://www.nc3rs.org.uk/) and through involvement in the EU VetBioNet project https://www.vetbionet.eu/best-practice-guidelines/.

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

I am registered with the NC3Rs website and I subscribe to the NC3Rs e-newsletter where the latest 3Rs developments are updated monthly, including relevant literature. Our institute has a 3R's

committee which focuses on advances in the 3Rs area which provides discussions and activities on 3R measures which ensures that we are up to date with recent advances in this area. In addition, all opportunities to include improvements to the 3Rs will be considered for each study.

3. Ischaemia reperfusion injury and organ transplantation

Project duration

5 years 0 months

Project purpose

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

Key words

Transplantation, Ischaemia-reperfusion Injury, Rejection, Treatment

Animal types	Life stages
Mice	adult, embryo, pregnant, juvenile, neonate
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 examine the mechanisms of organ injury in transplantation and to develop new therapeutic strategies, by studying ischemia (restriction in blood supply), the immune response and to investigate the efficacy of therapeutic interventions to improve acute and chronic organ 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?

Organ transplantation is a life-saving treatment for end-stage organ failure, but there is a growing disparity between the number of patients in need of an organ transplant and the number of suitable donor organs available. In January 2020, there were 6183 people in the UK currently waiting for an organ transplant. Conversely, the total number of patients

whose lives were saved or improved by an organ transplant fell by 2% in the same year. The total number of transplants performed also fell, ranging from a 3% decline for pancreas transplants to 20% for lung or heart-lung transplants. This is in spite of a 2% increase in the referral rate of potential donors, as well as a 1% increase in the overall consent rate for organ donation.

In the UK alone, approximately 1000 patients die or are removed from the transplant waiting list every year due to the shortage of suitable organs for transplantation. This is a gross underestimation of the need for transplant organs, because a large proportion of patients with end-stage or organ failure are never offered transplantation, a decision itself driven in large part by the lack of available organs. This shortage of organs has necessitated in the usage of less-than-ideal organs, including from donors in whom the organs are exposed to a period of ischaemia prior to transplantation. This results in relative impairment of the function of these organs after transplantation. This initial injury can result in delayed function of the organs after transplantation, as well as augmented immune response (the immune system is working harder than usual to fight off a disease or infection) to the transplanted organs; this also leads to reduced long-term organ function and organ failure, exacerbating the shortage of organs for transplantation. The most important anticipated benefit of the proposed work, therefore, is to improve the quality and quantity of organs available for transplantation, thus improving both the quality of life of transplant patients and reducing the number of patients dying on the transplant waiting list.

This project aims to improve our understanding of the cellular mechanisms that underlie ischaemia- reperfusion injury (tissue damage caused when blood supply returns to tissue) and test the safety and effectiveness of promising novel therapeutic agents. New treatments that reduce cellular injury and organ dysfunction will help overcome the national and international organ shortage by increasing the availability of organs suitable for transplantation. Improved rates of organ transplantation would have an enormous societal impact by shortening waiting lists, improving the quality of life of patients, and reducing complications and death from organ failure.

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

This project will generate academic knowledge, communicated in publications, that will support future research, new treatment products, and the basis of clinical studies which will have health and economic implications.

It is expected that the proposed project will ultimately enable novel therapeutic interventions (that may include drugs, cells or artificially created tissues) to improve acute and chronic organ function in transplantation. The work carried under the same project, that will continue in the new Project Licence, has already advanced one therapeutic agent from mouse and rat models to pig and human models, resulting in design of a human clinical trial. Importantly, because the underlying mechanisms of ischaemia-reperfusion injury in organs transplantation are shared with other common conditions such as myocardial infarction (supply of blood to the heart is suddenly blocked - heart attack) and ischaemic stroke (supply of blood and oxygen to the brain is blocked), the insights generated from this work will make a significant contribution to other clinical disciplines beyond transplantation.

The results of these experiments will be published in peer-reviewed journals and presented at national and international conferences. The treatments that are validated in the project may include new intellectual property. From our experiments, we hope to learn

what the best ways to deliver the treatments are, and how often and how much of the treatments need to be given. Altogether, these will generate a blueprint for clinical studies, which will bring successful new treatments to patients.

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

The outcomes from work carried out in this proposal will be of significant benefit to researchers in the fields of transplantation, ischaemia-reperfusion injury, immunology and beyond. We anticipate this will also result in direct benefit to clinicians and patients by enabling the clinical translation of these technologies and therapeutic approaches. Specifically, we anticipate the findings from this work will directly inform the design and conduct of clinical trials in transplantation by academics and pharmaceutical organizations. Throughout the project, researchers both inside and outside the field of transplantation will benefit from the above outputs. IR (ischemia-reperfusion) injury occurs in many human diseases, including myocardial infarction (heart attack) and stroke (when the blood supply to a part of the brain is cut off). The processes that occur in mitochondria (membrane-bound cell organelles), cells and organs are applicable to all of these diseases. Because the cellular processes underlying injury in transplantation, heart attacks and strokes are similar, we anticipate that our findings, including new drug treatments developed for use in transplantation, could potentially also be of benefit in treating heart attacks and strokes. Importantly, our raw data, publications and presentations will disseminate new knowledge to other researchers, who can then build on the discoveries.

In the short-term (1-3 years), the primary beneficiaries of the proposed project will be other researchers who are also developing new therapies in the fields of transplantation, ischaemia- reperfusion injury, immunology and beyond. We anticipate that the findings of this study will be of broad relevance to the research community in this field. In the medium term (3-5 years), we anticipate that industrial companies involved in the manufacture of therapies, who are essential for the ultimate production of these therapies, will also benefit from the findings of this study. In the long-term (5-7 years), this project will benefit patients. The enefit will initially be limited to those patients enrolled in clinical trials. We hope that ultimately (7-10 years) large numbers of patients will benefit from the findings of this study.

More specifically, we expect to generate at least one new therapy and advance it to a stage that it can be investigated further in large animal models. In the long-term (5-7 years), we expect that the findings of this study will result in the design of at least 1 human clinical trial to test the safety and efficacy of a novel therapy developed in this project.

During our work under the preceding licence, we have already started extending the findings of our studies on the mechanisms of ischaemia reperfusion injury to pig and human models. We have demonstrated that these findings are relevant to human hearts and kidneys, thus confirming that therapeutic interventions have a high chance of success in humans. Importantly, we have identified 2 drugs that reduce ischaemia-reperfusion injury in mice. We have already started testing these agents using pig and human organs.

The surgical and scientific techniques used in this project are very specialised, and therefore limited to a few experts who are able to carry them out. As this project progresses, more researchers will be trained in these specialist skills. They will go on to apply them in collaborative or new studies in the future, ensuring the dissemination of good experimental practice and model refinement.

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

We will disseminate all findings of our studies, including unsuccessful approaches, through publication in peer-reviewed journals, presentation at scientific conferences, and through meetings with other researchers. All publications will be open access, including through platforms such as F1000Research. This project includes collaborations with a large number of researchers with expertise in complementary areas, and this network will be utilised to maximise the dissemination of the new knowledge gained through this project.

Species and numbers of animals expected to be used

- Mice: 5375
- Rats: 100

Predicted harms

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

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

The project will utilise normal wild type or genetically modified mice. Mice are the lowest species with a comparable physiology that enables the study transplant organ injury induced by ischaemia and the immune response (reaction which occurs within an organism for the purpose of defending against foreign invaders) and to investigate the efficacy of therapeutic interventions to improve acute and chronic organ function. Importantly, genetic strains of mice are available that allow studies to be designed that can generate valuable information about specific therapies. In one of the experiments we may use rats as their larger size makes the complex surgery easier and more likely to be successful.

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

Animals are typically expected to undergo only 1 or 2 surgical procedures under general anaesthesia from which the animal will be recovered. If >2 invasive (ie, laparotomies and excluding re-suturing of wounds, injections or bleeding) general anaesthetic procedures are planned (excluding procedures under terminal anaesthesia), the additional invasive general anaesthetic procedure will only be undertaken if the animals do not display signs of ill health and after discussion with the named veterinary surgeon.

Two experimental models will be used to study ischaemia-reperfusion injury (IRI) in the kidney. In the first model, blood vessels to one or both kidneys will be clamped under general anaesthesia for up to 90 minutes, followed by reperfusion. Animals will be recovered and maintained for up to 9 months to investigate the long-term impact of IRI on kidney function. The duration of general anaesthesia will not exceed 6 hours. In the second model, recipient mice will first undergo removal of one kidney (nephrectomy) under general anaesthesia. A donor kidney that has been exposed to various periods of ischaemia, will then be transplanted into recipient animals during the same operation and reperfused. Animals will be recovered and maintained for up to 9 months to examine long-term organ function. In some kidney transplant recipient animals, the remaining native kidney of the recipient animal will be removed under general anaesthesia no less than 5 days after transplantation. The aim of the removal of the second native kidney is to enable the function of the transplanted kidney to be monitored independently of the native kidney. The second native nephrectomy will only be performed if the animal has fully recovered from the transplant operation. By transplanting donor kidneys to genetically identical or

mismatched recipients, the impact of IRI on the immune response to transplanted kidneys can be studied. Blood sampling and imaging may be performed to assess injury to the kidney.

To examine IRI in the heart, donor hearts will be transplanted into the abdomen of recipient animals and reperfused. Heterotopic heart transplantation (heart transplant to abnormal place) is an experimental technique that is optimised and routinely performed by our group. It enables the transplanted heart to re-commence beating in the abdomen after reperfusion. Importantly, because the heart is transplanted heterotopically, subsequent failure of the transplanted heart (for example through acute or chronic rejection) does not lead to ill health in the recipient animal. Moreover, simple palpation can be used to non-invasively assess the function of the transplanted heart through the abdominal wall. By transplanting donor hearts to genetically identical or mismatched recipients, the impact of IRI on the immune response to transplanted hearts can be studied. Animals will be kept up to 9 months after transplantation to examine long-term organ function. Blood sampling and imaging may be performed, in addition to palpation of the abdomen, to assess injury to the transplanted heart. Animals receiving a heart transplant may be treated with cells or drugs (for example, to modify the immune compartment), have blood sampling or non-invasive imaging prior to the heart transplant. The heart transplants will be performed under terminal anaesthesia if the period of reperfusion is less than 60 minutes and during which other manipulations such as imaging may be performed. The duration of general anaesthesia will not exceed 6 hours. Such a short period of reperfusion may be necessary to study organ injury soon after reperfusion.

To examine IRI in the liver, the blood vessels supplying the whole liver or part of the liver will be clamped under general anaesthesia for up to 90 minutes, followed by reperfusion. The duration of general anaesthesia will not exceed 6 hours. Animals will be recovered and maintained for up to 9 months to study the long-term impact of IRI on liver function. Blood sampling and imaging may be performed to assess injury to the liver. The liver IRI experiments will be performed under terminal anaesthesia if the period of reperfusion is less than 60 minutes and during which other manipulations such as treatment with drugs or imaging may be performed. Such a short period of reperfusion may be necessary to study organ injury soon after reperfusion.

To examine vascular rejection, donor or bioengineered aorta grafts may be transplanted orthotopically (same anatomic location as the original tissue) into the abdomen of mice or rats under general anaesthesia. Animals will be recovered and maintained for up to 9 months to study the long-term function and rejection of aortic grafts.

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

It is expected that most (more than 90%) of animals will recover rapidly post-surgery.

In some cases, due to the surgical procedure, some the animals may experience weight loss, reduced food intake, reduce movement or an abnormal coat (piloerection and/or wet ungroomed coat). In such cases, the animals will be humanely killed, if these clinical signs do not respond to treatment (such as high energy and easily digestible diet) and persist for up to 24 hours.

When rapid adverse effects may be expected, animals will be monitored very frequently (up to one hourly) during the initial period (approx.6 hours) when adverse effects are most likely to occur (based on data from previous animal experiments and clinical studies).

Animals will also be humanely killed if they experience clinical signs that approach the limits described in the project according to the Home Office guidelines and as stated in the protocols.

Expected severity categories and the 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 25%, moderate 75%, severe 0%.

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

- Killed
- Used in other projects

Replacement

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

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

The definitive investigation of mechanisms and therapy of ischaemia-reperfusion and immune injury during transplantation requires study in intact animals. Transplantation of organs into animals are necessary to enable monitoring of acute and chronic organ function. Much of the proposed work is carried in the laboratory and using human tissue or organs only (including perfusion with blood on specialised machines), thus minimising the need for animal experimentation. Importantly, it is anticipated that this work using isolated human organs will lead to the refinement and optimisation of IRI models which can be ultimately used to replace experimental use of animals.

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

In the past we have established the Biorepository for Translational Medicine which aims to provide fresh normal human tissue from deceased transplant organ donors for research, including for study ischemia-reperfusion injury in organ transplantation. During this project, we will continue to use human tissue and organs, exposed to a range of ischaemic conditions to study the impact of ischemia on mitochondrial, cellular and tissue viability. This enables the use of human, rather than animal tissues/organs. Additionally, this programme supports more than ~50 research projects locally by providing access to live human tissue/organs for research. Many of these research projects would have previously only been possible using animal tissue. This programme continues to make a significant contribution to Replacement.

Further to the above, to examine the ischaemia-reperfusion injury in organ transplantation, we will continue to use and further improve the ex vivo (outside of the living body) normothermic (normal body temperature) perfusion technology, developed in our lab, in which human organs are perfused with ABO blood-group-matched oxygenated blood. This technique enables the PPL holder to use human organs to study the IRI. This approach has enabled the clinical translation of our previous work towards human clinical trials and

make significant contribution to Replacement of animals with human organs to achieve in some experiments, the same aims of this project.

In general this project has been preceded and informed by experiments using cells grown in special dishes in the laboratory (in vitro) and donated human organs that have been declined for clinical transplantation. Non-animal alternatives have, therefore, been extensively used as far as possible, in order to replace animal experiments.

Why were they not suitable?

We are using all of these alternative approaches to reduce the number of animals used in the proposed project. Use of cells does not enable the complexities of organ injury during transplantation to be assessed fully. While we have and continue to make extensive use of human organs, these are very rare and access to them is very unpredictable. Moreover, because some of the human organs are of less-than-ideal quality (which is why they were not used for transplantation), they are not always able to be used to generate robust and reproducible data. Lastly, ex vivo experiments using human organs cannot be used to characterise the chronic aspects of organ injury and the efficacy and safety of novel therapeutics on a long-term scale. The definitive investigation of the mechanisms of organ injury in transplantation and to develop new therapeutic strategies requires an intact and functional immune system in an animal model.

Reduction

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

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

The number of animals have been estimated based on the range of studies that are planned, as well as based on the previous similar studies we performed during the last five years. Based on our previous experience, we are able to predict, for each study, the number of animals that are required to generate reliable and reproducible data. Using our previous experience, we are also able to predict how many studies we can perform in a given time period.

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

Our in-vivo experiments will continue to be designed to include the minimum number of animals needed per study, that can lead to reliable conclusion. The PPL holder monitors the number of animals used in each experiment and approves the study plans to meet the requirements of Reduction of animals. In order to minimize the number of animals used, the cohort sizes are determined with attention to the experimental variability in the data generated in each specific model, with aim of obtaining statistically and biologically-significant data using fewest animals as possible. We have a wealth of previous data to show that a group size of 5-6 individual animals are usually appropriate to allow definitive conclusions to be drawn using the minimum numbers of animals. When a new therapy is under investigation, we will first perform pilot experiments with small animal groups (typically 2-3 animal per group) to confirm the appropriateness of the experimental design

and to generate pilot data to enable group sizes to be formally calculated. All experiments are conducted using randomisation and blinding as far as possible. Littermate animals will be randomised to receive experimental or control treatments to minimise bias from age, sex and weight. Data analysis is routinely conducted in blinded manner to minimise bias.

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

Many of the experiments will be designed to enable important data to be generated from individual animals about the impact of ischemia on multiple tissues, for example, when donor animals are treated with therapeutic agent, the kidney, heart and liver from the same animals are retrieved and tissues simultaneously analysed to generate important insights about injury to all tissues. This results in Reduction in the number of the animals used in this project by collecting more data from the animals that are already used and in order to maximise their use and reducing the need to use more animals.

Furthermore, in addition to the in vivo experiments (experiment on living organism), we have an advanced and comprehensive programme that uses cell lines and in vitro culture systems to examine the efficacy of agents to reduce the ischemia reperfusion injury during the transplantation. For example, these in vitro experiments are used to screen potential therapeutic agents and identify the likely therapeutic window of the drugs. This approach resulting of significant reduction of number of the animals been used.

All tissues obtained from animals will be, where possible, shared with other researchers to maximise data generated from the experiments. This will include tissue from experimental animals that have been killed, as well as animals used for breeding and surplus to requirement. Data generated from the experiments, including raw data, will be made available to other researchers in order to maximise the benefit of the experiments.

Refinement

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

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

The experimental models and techniques that will be used in this project are: Kidney or liver blood vessel clamping - ischaemia and reperfusion

Kidney or liver pedicle clamping - Non-recovery ischaemia reperfusion injury Organ and tissue donor

Organ and tissue donor: Non-recovery Kidney and Heart transplantation Blood-Vessel Transplantation

All experimental models have been refined to ensure they cause the least pain and suffering. Importantly, none of the procedures are expected to result in severe clinical signs (such as persistent abnormal behaviour or persistent weight loss). Animals will be

humanely killed if they display clinical signs that do not respond to treatment (such as easily digestible food or pain relief medication). Animals will therefore not be permitted to experience lasting harm.

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

Mice are the least sentient animals that can be used to generate valuable data to investigate the transplant organ injury induced by ischaemia and the immune response and to investigate the efficacy of therapeutic interventions to improve acute and chronic organ function. As the immune response and the action of potential therapeutic strategies takes days to weeks to manifest, experiments cannot be performed exclusively under terminal anaesthesia. Due to the small size of mice, rats may need to be used for transplantation of vessels to ensure technical surgical feasibility.

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

We have refined all our surgical procedures, including transplantation of heart and kidneys. We have developed robust and effective methods to instruct and supervise new researcher in these methods and our expertise is regularly sought by other groups to perform procedures in order to reduce rates of technical failure. We have also provided the infrastructure and trained staff and supervision to ensure success of the technical procedures are as high as possible. All experiments are performed using aseptic techniques and we have not had documented cases of surgery-related infections in recent years. Experiments are designed so as to minimise the need to perform experiments out of normal working hours. The experiments in this project have been designed so that death of animals is never an expected endpoint. No animals will undergo procedures that are routinely expected to cause severe ill effects, thus limiting the distress and discomfort experienced by the animals.

In close collaboration with the staff at our animal facility, we have a robust mechanism for the post- operative monitoring of our experimental animals. Animals are also given routine post-operative pain relief medication, which has been proven to be effective in previous similar studies. When adverse effects may be expected, we readily increase the frequency of monitoring to identify animals that may be experiencing adverse effects. We also have our own dedicated animal technicians who ensures that our animals receive close attention if there are any concerns. We have achieved a number of significant refinements during the previous series of studies. These include enhanced environmental enrichment (such as extra card-board housing) and use of high-energy or tasty diets to prevent weight loss. Where adverse effects may be expected, we will perform particularly close and frequent monitoring of animals, including through the use of observation sheets and body weight records. We will continue to strive to develop new refinements.

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

All experiments will be conducted and reported in adherence to best practice guidelines including those published by the Laboratory Animal Science Association (LASA), such as guidelines for record keeping, performing surgery, education and training, and reporting of experimental results. We will also follow the PREPARE guidelines, so we can ensure that our animal experiments are conducted in a more refined way and that animal welfare is prioritized throughout the research process.

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

As a current project license holder, I am closely involved with the activities of the 3Rs committee at my institution, including the development of recommendations and dissemination of information relating to advances in 3Rs. I intend to continue with my activities, included but not limited to:

Stay up-to-date on the latest developments and best practices in 3Rs by regularly reviewing resources and guidance from organizations such as NC3Rs and Norecopa guidances: NC3Rs Resource Hubs, Norecopa Databases and Guidelines, NC3Rs Training and Norecopa Network

Incorporate 3Rs principles into the research design and planning process. This may involve considering alternative methods to animal testing, minimizing the number of animals used, and ensuring that animal welfare is a top priority throughout the research project.

Engage with other researchers, stakeholders, and experts in the field to share knowledge and best practices. Attend conferences and workshops, participate in online forums and collaborate with other researchers to stay up-to-date on the latest advances in 3Rs.

Continuously evaluate and refine our research methods to ensure that you are incorporating the most effective 3Rs practices. For example: assessing the effectiveness of alternative methods, and incorporating feedback from animal welfare experts and ethics committees.

4. Germinal Centers in Infection and Cancer

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Germinal center, Infection, Immunisation/Vaccination, Cancer, Ageing

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?

Germinal centers are a critical component of the immune system from which antibody producing cells are generated. In this project we aim to investigate and compare the formation and function of germinal center reactions across health challenges across life stages.

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

Why is it important to undertake this work?

Recent world events in the form a SARS-Coronavirus-2 pandemic have yet again demonstrated the critical need to investigate the immune response to infection and to gather understanding on how severe disease can be prevented by immunisation/vaccination. The germinal center reaction is a critical component of the immune response from which antibody producing cells are generated and although the research proposed in this project is basic in nature, it has inherent translational potential. This is in part because the infection and vaccination settings we have established in the laboratory closely mirror relevant clinical situations that are of high importance for public

health. As consequence the understanding of germinal center dynamics and role is key to uncover mechanisms that may allow to tailor this reaction to optimise vaccination protocols that enhance antibody mediated pathogen clearance.

More recently it has been recognised that germinal center reactions play a critical role also in controlling cancer development and progression. However, how germinal center reactions are formed in a cancerous scenario and what components of the reaction are relevant in the anticancer response remain unknown. A better understanding of these components may unleash truly novel therapeutic strategies for haematological cancers, such as lymphoma and multiple myeloma, and solid cancers, such as breast, skin, lung and pancreas. The cancer models established in the laboratory and used in this project are of pre-clinical importance and of value for the development and testing of anti-cancer therapeutics, including therapies aiming to stimulate germinal center reactions that may lead to the elimination of cancer cells. The information gathered in these studies may thus also allow a better definition of the risk of cancer formation, progression and relapse and assist the development of new biomarkers and novel therapies, relevant for the design of future cancer treatments by the pharmaceutical industry.

As individuals age, changes in the immune system often mean that older individuals lack long-term protective immunity following infection or immunisation/vaccination, as recently shown for SARS- Coronavirus-2. While cancer can affect people of all ages, the risk of developing cancer increases significantly as we age. This is because ageing is associated with a gradual accumulation of DNA damage and other molecular changes such as genetic mutations that can lead to cancer. In addition to genetic mutations, ageing is also associated with changes in the immune system that can make it less effective at recognising and destroying cancer cells. This may contribute to the increased incidence of cancer in older adults. In this project we will investigate the impact of ageing in germinal center reaction dynamics and function. The overall expectation is to gather age-related biological data on germinal center reactions and devise ways for interventions that offer enhanced longer-term protective immunity to older individuals.

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

The work in this project aims to understand the dynamics and role of germinal center reactions in infection, immunisation/vaccination, and in cancer. We also aim to explore the impact of ageing in the dynamics and function of germinal center reactions in these contexts. As such we will gather new information that will assist:

- the improvement and design of future immunisation/vaccination regimens,
- the improvement and design of novel anti-cancer therapies, including those that provide an avenue for treatment of patients that relapse or are refractory to current standard treatment,
- develop therapeutic approaches that can be helpful in mitigating immune related changes and promote healthy ageing.

The findings obtained through this project will be output to the wider scientific community as research presentations and publications, in both open access repositories and peer-reviewed publications.

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

This project will aid to uncover the dynamics and role of germinal center reactions in immunisation/vaccination, infection and cancer, an active area of research and a long-term goal of the laboratory.

Basic science and the scientific community: In the shorter term the benefit is to increase the knowledge on these subjects for the scientific, industry, funding agency and patient community. Our work will also generate new tools that can be used to interfere/manipulate the germinal center reaction. The tools generated from our work will be available to other researchers to be used and inform their own work.

3Rs: In the longer term, our work holds great promise for helping to reduce the number of animals required for future germinal center reaction research. Our proposed work will collect large datasets, including single cell and RNA sequencing that can be further analysed by other researchers before animal experiments are performed. In turn, these large-scale data will also help us refine our own hypotheses and design more targeted experiments that will enable us to achieve our scientific objectives using a smaller number of animals in the future. Similarly, once validated, the methodologies used have the potential to either decrease the numbers of animals needed to obtain similar insights or obtain more data-rich results from each animal without repeated sampling.

Clinical translation/patients: Ultimately, we envision that our work will benefit human patients by improving our understanding of the germinal center reaction and its impact on infection, vaccination and cancer. Thus, in the longer term the findings derived from the studies in this project will impact the development of new therapeutics, and patient clinical management, with the expectation to improve patient care and responses to treatment.

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

The outputs will be maximised first through interactions, sharing of data and of mouse models with colleagues in the scientific community to gather further insight and avoid repetition. Negative results will be presented and published as far as possible. This should minimise unnecessary repetition by colleagues. The work in this project will also be disseminated through presentations to funding agencies and patient advocate groups, and in scientific conferences and by publication as readily and free of charge accessible pre-prints and in peer-reviewed journals. Additionally, we have fostered productive relationships with various stakeholders from diverse sectors, such as those involved in industry and healthcare settings. Our primary objective in these collaborations is to facilitate the translation of research findings into practical applications, bridging the gap between laboratory experimentation and real-world clinical implementation. We also aim to achieve maximal dissemination of the results in this project to health policy makers and the public through all available channels.

Species and numbers of animals expected to be used

- Mice: 60000

Predicted harms

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

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

Despite the differences, the mouse is one of the model organisms that most closely resembles humans. The human and mouse genomes are approximately the same size and display virtually the same number of genes, which are for the most part functionally conserved. Further, mice have genes not represented in other animal model organisms (e.g., Caenorhabditis elegans i.e., Nematode worm, and Drosophila melanogaster i.e., fruit fly) such as those involved in adaptive immunity and required for germinal center reactions to take place. Mice and humans largely display conserved cellular and molecular changes with age, despite these occurring at different timescales. Of note, aged mice and older humans both show impaired immune responses to immunisation/vaccination and infection that has been linked to reduced protection from subsequent infection. Similarly, to humans, aged mice have an increased propensity for cancer development. Mice can be genetically altered, and there is extensive literature concerning the topics of investigation in this project. Further, our own studies can be enhanced by combination with many complementary mouse models developed by colleagues in the field of research. In studies of the immune system and cancer, the topic of the present proposal, the mouse has played a central role in the discovery of function of genes and mechanisms. Although some of these characteristics apply to other mammals, the mouse shows important advantages as a laboratory model over other mammalian organisms e.g., it is small and easy to maintain in a laboratory setting and their breeding cycle is short. In addition, mice produce between 5-10 offspring per litter, which makes them suitable for genetic experiments.

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

A fraction of mice will be injected with components used in vaccination or infected with virus, such as influenza, known as flu, and SARS-Coronavirus-2, known as Covid-19, to investigate the formation and function of the germinal center reaction. In the larger fraction of mice in this project a mouse will experience these injections and infections only once in their lifetime; however, the scientific objectives of the proposed project require on occasion the need to mimic vaccination boosters and re-infection.

To understand the formation and function of the germinal center reaction in cancer, a fraction of mice will be developing cancer, such as lymphoma, breast, lung, and pancreas, that can either occur spontaneously due to the genetically engineered composition of the mouse or through the injection of tumour cells orthotopically or subcutaneously. Treatments, including chemo- and radiotherapy, other drugs and antibodies may be employed in these settings with the aim of discerning the role of various cell populations of the immune system as these lead to their depletion.

Surgical procedures to perform partial or complete removal of the spleen or of lymph nodes may be performed to gather knowledge of the broader role of the immune organs where germinal center reactions take place. Partial or complete removal of the cancer by surgery may be performed to investigate processes of cancer driven formation of germinal center reactions. Despite these possibilities mice will undergo no more than two surgeries requiring the exposure of internal tissues throughout the course of their lifetime.

Some of these procedures may be performed in aged mice. The study of mice at different ages, using longitudinal approaches over the lifetime within the same individual or cross-sectional approaches in independent individuals are necessary to determine whether the mechanism being investigated is age- independent or age-dependent.

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

In the proposed studies relating to infection and immunisation/vaccination mice may present adverse effects including weight loss, hypothermia, laboured breathing, diarrhoea, bloody stools, hunched position, lack of movement, all to varying degrees. For all models, we have detailed knowledge of the days of highest severity of adverse effects may occur, which depending on the infectious agent, immunisation/vaccination regimen and mouse strain generally may last between one and four days. At the defined critical time-points for each experimental condition mice will be monitored daily, using a score sheet (Paster et al., 2009: PMID 19619413) and/or weight loss as the main parameters. If weight loss is used as a parameter any animals experiencing more than 15% weight loss between the since the start of infection, or immunisation/vaccination, will then be weighed daily and killed if the 20% weight loss is reached. Once the critical days are overcome, mice that recover rapidly are undistinguishable from uninfected mice, like in human infections. Only a minority of mice will undergo a full-time course of infection i.e., from infection to recovery or humane endpoint, while most mice will be killed at predetermined time points to study immune parameters.

With respect to cancer studies, in most cases animals used in this license will form tumours. The size and impact of these tumours on the animal will differ between model and method. Some cancer cells might grow well only within a unique site and the likelihood that they will spread to other areas of the body is low, therefore the degree of severity of adverse effects is minimal. Other cancer cells may spread to other areas of the body therefore leading to varying degrees of adverse effects. We have in depth knowledge and experience on all the models that we use, both transplantation and spontaneous, and can predict well the time frame over which the animals will not show adverse effects. Adverse effects may, however, include weight loss, hypothermia, laboured breathing, diarrhoea, bloody stools, hunched position, lack of movement.

The use of interventions that modulate the immune system such as ablation of cells of the immune system can make the adverse effects less predictable, but the animals will be monitored in this case to minimise any suffering and in most cases, we will terminate experiments before the onset of undesirable adverse effects.

Surgical procedures will be chosen to minimise perioperative pain, recovery time and complications. Analgesia will be provided according to contemporary best practice. Mice will be monitored for at least 4 days after surgery and weight will be recorded daily then twice weekly. Animals are expected to make a rapid and unremarkable recovery following surgery.

For studies involving aged mice the risk of adverse effects is higher. However, multiple studies have shown that it is feasible to develop a reliable system to measure the index of frailty in ageing mice in a non-invasive manner (Wilkinson et al., 2019 PMID: 31403890). These measures will start being used once mice reach 15 months of age as suggested by the authors, with the aim of limiting the risk of unexpected adverse effects, further compromising already challenged animals.

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

What are the expected severities and the proportion of animals in each category (per

animal type)?

The expected severities predicted by the proposed work to address the scientific questions are mild or moderate with the following breakdown of proportions:

Mild (60%)
Moderate (40%)

When investigating germinal center reaction mechanisms in conditions mimicking immunisation/vaccination regimens the expected severities will be mild to moderate, with the larger fraction of mice experiencing only mild adverse effects. Where infection studies are performed, mice will mostly experience moderate adverse effects. Depending on the scientific questions asked during infection, we have observed that on occasion mild adverse effects show a higher interindividual variability, making the interpretation of results difficult, leading to the requirement of a higher number of mice to be studied per group. Initial comparisons will tell us whether this is the case, and the mildest possible protocol to model human disease will always be applied. We will weigh up in each individual experimental setting which is the earliest time point possible to assess crucial immune parameters.

For work addressing cancer growth it is expected that the greater proportion of mice will reach moderate severity, due to repeated treatment procedures, such as anti-cancer substance administration, or tumour burden. Cancer is a complex disease, however the knowledge of all the tumour models used in this proposal allows a good prediction of the time frame of the disease progression. Therefore, mice will be humanely killed mostly based on a predefined experimental time-point rather than on clinical signs i.e., an experimental end point that is time controlled will occur before a humane endpoint as determined by deterioration of heath conditions.

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?

Valuable studies of human immunology and cancer are performed using patient material; however, in the context of the germinal center reaction mechanistic understanding requires the use of living animals.

The development and function of the immune system involves many different cell types interacting in a dynamic three-dimensional environment.

Similarly, cancer development and spread involves a plethora of interactions between cancer cells and their surrounding cells, governed by multiple signals originating from both their immediate neighbours and from distant tissues.

Mice and humans largely display conserved cellular and molecular changes with age,

despite these occurring at different timescales. Of note aged mice and older humans both show impaired immune responses to vaccination and infection that has been linked to reduced protection from subsequent infection. Similarly, to humans, aged mice have an increased propensity for cancer development.

These processes cannot currently be fully recapitulated in vitro even using the most sophisticated protocols.

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

We have collected as much evidence as possible from existing literature, and through the analysis of available human and mouse infection, immunisation/vaccination and cancer datasets, including e.g., RNA sequencing and Exome sequencing (e.g., GEO, EGA, NCBI). These analyses are, whenever possible, confirmed using primary patient material and/or human and mouse cell lines. These studies have preceded and guided the generation of relevant mouse models.

Why were they not suitable?

Human and mouse datasets provide information on possible important correlations but are insufficient to establish causation. Even the most recently developed and highly sophisticated in vitro models are not representative of the complexity of cellular interactions in the immune system and in the cancer microenvironment. Lastly, the study of cancer cell lines in vitro does not allow to investigate the dynamics and function of the immune system, such as germinal center formation 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?

Given that the proposed work on this project is a continuation of ongoing work in the laboratory, the number of animals requested considers detailed information from the previous 10 years. Not all animals will be given immunisation/vaccination or develop cancer, e.g., a fraction of mice will be used to collect tissues only.

We aim to use the minimum number of mice per group that will be informative, however, it is important to mention that in the case of sporadic cancer models there is low penetrance and larger cohorts are needed to obtain significant results.

When studying aged mice, the JAX laboratory (https://phenome.jax.org/projects/Yuan2) and others (Kunstyr et al, 1976 PMID: 977921) have that 20 to 50% of mice die of natural causes that are unrelated to their genotype or experimental conditions. This knowledge needs to be taken in consideration when calculating the number of mice required to achieve the work proposed in this project.

What steps did you take during the experimental design phase to reduce the number

of animals being used in this project?

For all experiments, we have long-standing experience in experimental design and optimal handling to reduce operator-dependent variance.

A variety of strategies are used during the experimental design phase to reduce the numbers of animals used in the project, e.g., we are aided by the NC3Rs Experimental Design Assistant and use the Planning Research and Experimental Procedures on Animals: Recommendations for Excellence (PREPARE) guidelines (https://norecopa.no/PREPARE). In combination we consult statisticians and the bioinformaticians to advise on the minimum number of animals required to achieve the goals of the work proposed in the project licence.

In most cases we use blinded studies and include several conditions in separate treatment groups in the same experiment, so to reduce animal usage as untreated controls and make full use of multifactorial design to enhance statistical power.
We routinely perform experiments on both males and females to make full use of the mouse colony and to avoid sex-bias in our results and to discover sex-specific effects. An exception is when performing experiments related to breast cancer. In this case we will mostly use females because, breast cancer incidence is predominant in females, with most of the available datasets in the human do not including male subjects. There is also the lack of experimental systems, namely the availability of cell lines for transplantation studies.

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

We maximise the amount of data collected from each mouse through the analysis of multiple organs and by taking part in a tissue sharing initiative at the institute.

We also store many organs from experimental mice in case novel findings or future research directions require analysis of these organs.

Also, when possible different treatment groups (e.g., mice treated with control or depleting antibodies) will be co-housed in the same cage, to avoid artefacts driven by differences in microbiota composition.

Where genetically modified mice are tested, litter mates will be used for similar microbiota and to avoid artefacts due to minor differences in the genetic background. Where the use of littermates is not possible, co-housing prior to experiments or exchange of cage bedding will be considered. This is easier for females but can be achieved for males if they are co-housed immediately after weaning, thus requiring early genotyping or homozygous lines. Co-housing will be particularly challenging for ageing experiments with males, given that in-cage fighting often requires separation of males.

We mostly breed the genetically altered animals that we use ourselves to be able to respond to the experimental needs by prompt and often transitory colony size adaptation. This reduces waste from overbreeding. Obtaining non-genetically altered mice from in house facility-shared breeding allows better efficiency for larger colonies.

Education and training are under constant update for those working under this project license to ensure and improve animal welfare and minimise operator-dependent variability

in results.

We often use small pilot studies to estimate the effect size and the directions for future experimental settings.

When using genetic engineered models, we take advantage of the most refined systems currently available to the scientific community. In that regard we take lead from the Home Office guidelines with respect to the breeding of genetically altered mice and take decisions to archive lines by cryopreservation when not required over a period, as per recommendations in the link below.

(https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/773 553/GAA_Framework_Oct_18.pdf)

Refinement

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

Which animal models and methods 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 proposed in this project uses both non-genetically altered and genetically altered mouse models. We perform studies of the immune system in these mice and their development of spontaneous cancers. We generate transgenic mice in which mutations are induced specifically and conditionally using e.g., Cre-LoxP conditional alleles, or alleles, which function can be activated or terminated using Tet-On and Tet-Off systems, e.g., CCSP-rtTA, TeO-EGFPL858R, these transgenic mice develop lung tumours only upon provision of doxycycline therefore avoiding adverse effects of constitutive oncogene expression.

In addition, we take advantage of cell and cancer cell transplantation techniques to generate orthotopic, subcutaneous models of cancer. These include B and T cell lymphoma, multiple myeloma, breast, lung and pancreatic cancer. We strive to minimise surgical methodologies for the injection of cells using ultrasound guided injection techniques, drastically reducing adverse effects in the mice.

When performing studies to understand the impact of the removal of organs of the immune system we aim to remove lymph node/s rather than the spleen, as it is a less invasive surgery.

We actively seek to identify immune parameters to refine to avoid dependence on clinical scores.

We primarily use standard routes of administration such as intravenous or intraperitoneal injections. The active concentration, volume, stability, and toxicity of a particular substance may require its administration through a non-standard route. These include direct injection in the spleen, thymus, bone marrow (long bones), liver, intratumorally or peritumoral.

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

The mouse is one of the model organisms that most closely resembles humans. The human and mouse genomes are approximately the same size, and display virtually the same number of genes, which are for the most part functionally conserved.

Further, mice have genes not represented in other animal model organisms (e.g., Caenorhabditis elegans i.e., nematode worm, and Drosophila melanogaster i.e., fruit fly) such as those involved in adaptive immunity.

In other animals such as fish, germinal center reactions have not been characterised, and their immune systems are not comparable to those in humans.

Mice can be genetically altered, there is extensive literature concerning the topics of our investigation, and our own studies can be enhanced by combination with many complementary models developed by other researchers in the field.

More immature mice are not an option as embryos and newborns have only an immature if any immune system.

Because germinal center reactions take days to weeks to develop it is not possible to perform the studies proposed in this project under terminal anaesthesia.

Lastly, we need a physiology in our in vivo models which is as close as possible to humans including the study of across ages in an analogous manner to humans across the life course.

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

Whenever possible we generate transgenic mice in which mutations are induced specifically in a tissue and conditionally i.e., the genetic alteration is not present in the whole body of the mouse and throughout all its life course. This is achieved using e.g., Cre-LoxP conditional genes, or genes, which function can be activated or terminated using Tet-On and Tet-Off systems. As consequence mice do not display a phenotype until the immune or cancer associated mutation in the candidate gene is manipulated.

Where the immune status of the animals might compromise health, they will be maintained in IVCs (individually ventilated cages) under a barrier environment, to avoid infections.

In our experiments we will set clear humane endpoints and for each experiment, as part of good laboratory practice, write an experimental protocol, which will include details of possible adverse effects. These experimental protocols will be provided to all the staff involved in the experiment.

When considering which route of administration of substances to employ, we will strive to use the least invasive route whilst maintaining direct control of dose. Such as to activate a lymph node, instead of footpad injection, we choose to immunise mice with subcutaneous injection on the plantar surface, which causes less pain to the animals.

All surgical work in this project will be undertaken in accordance with the principles set out in the LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery or other

such publication promoting best practice. Surgical procedures will be chosen to minimize perioperative pain, recovery time and complications, e.g., preferring the use of lymph node excision to splenectomy where the experiment allows. Analgesia will be provided according to contemporary best practice and advice from the NVS/NACWO. Mice will be closely monitored for at least 4 days after surgery. Weight will be recorded on these days after surgery, then twice weekly.

For the administration of substances and cells the route used should be such as to achieve “best practice”, that is to minimize or avoid adverse effects, while minimising the number of animals used, and maximising the quality and applicability of results. For that reason, we propose in this project licence a variety of routes of administration of substances and cells to achieve the scientific objectives, while minimising the waste of mice.

We have detailed knowledge of the days of highest severity of adverse effects may occur, which depending on the infectious agent, immunisation/vaccination regimen and mouse strain generally may last between one and four days. At the defined critical time-points for each experimental condition mice will be monitored daily, using a score sheet (Paster et al., 2009: PMID 19619413) and/or weight loss as the main parameters.

For studies involving aged mice the risk of adverse effects is higher. However, multiple studies have shown that it is feasible to develop a reliable system to measure the index of frailty in ageing mice in a non-invasive manner (Wilkinson et al., 2019 PMID: 31403890). These measures will start being used once mice reach 15 months of age as suggested by the authors, with the aim of limiting the risk of unexpected adverse effects, further compromising already challenged animals.

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

We will follow the guidance given in the NC3Rs ‘Resource Hub’ (https://nc3rs.org.uk/resource-hubs) for example on blood sampling (https://www.nc3rs.org.uk/blood-sampling-mouse) and effective use of genetically altered mice (https://www.nc3rs.org.uk/GAmice).

We will also refer to the National Cancer Research Institute guidelines on using animals in cancer research published by Workman et al. 2010 (British Journal of Cancer 102, 1555 – 1577).

For surgery, we will follow the LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery (https://www.lasa.co.uk/wp-content/uploads/2018/05/Aseptic-Surgery.pdf).

With regards to infection models, we take advice from the "Considerations for Infectious Disease Research Studies Using Animal " (Colby et al, 2017 PMID: 28662751).

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

Updates on 3Rs and on new technologies are received on a regular basis from within the establishment and from NC3Rs and NORECOPA. Whenever we are able to refine techniques without impacting the scientific validity of the work, we aim to implement these advances.

5. Genes and immune cell function in health and disease

Project duration

5 years 0 months

Project purpose

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

Key words

Autoimmunity, Infection, Disease mechanisms, Genetics, Therapy

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The overall aim of this project is to better understand the biological processes that lead to the development of immune-mediated diseases, such as Crohn's disease or rheumatoid arthritis. In addition, we will investigate whether these processes might also affect immune responses to infection or cancer, which will help us determine whether they could be targeted therapeutically – either for treatment of autoimmune disease 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?

In the last 20 years we have seen large increases in the incidence of autoimmune

diseases. Currently, in the UK, there are nearly 5 million people living with an autoimmune disease, which is nearly double the number of people living with cancer, and this is only going to get worse. The incidence of most common autoimmune diseases is still rising at a rate of between 3 and 9% per year. To put this in context, a 5% rise in the incidence rates for all cancers has taken 10 years.

Many autoimmune diseases, such as Crohn's disease, type 1 diabetes and rheumatoid arthritis, develop in young adults and produce debilitating symptoms that can disrupt an individual's ability to hold down a job, complete education, form relationships and have a family. Autoimmune diseases are currently incurable, and although immunosuppressive therapy may be effective in controlling some of these diseases, these treatments can themselves be associated with significant side effects – compounding the health problems that many patients experience. For this reason, almost all immune- mediated diseases need better therapies, but the failure rate of drugs entering clinical development is high, with many candidate treatments ultimately proving ineffective.

So why are we in this position? The simple answer is that we don't understand these diseases well enough. Inflammatory bowel disease (IBD), for example, involves interactions of multiple components of the host immune system with the intestinal epithelial barrier and with viruses, fungi and bacteria in the gut – all of which needs to be triggered by poorly understood environmental factors and to occur in genetically susceptible individuals in order for disease to develop. However, this broad-brush overview hides the fact that many of the details remain unknown. For example, we don't know what goes wrong when the disease first develops, we don't know why some patients respond well to treatments and others don't, and we don't know what pathways are driving disease when treatments don't work.

So how can we understand these autoimmune diseases better? One of the best opportunities has been provided by genetics. Thanks to large-scale genome-wide association studies (GWAS), we now know of hundreds of regions of the human genome that are directly involved in one or more forms of autoimmune disease, with many of these regions also affecting susceptibility to infection. This not only provides a unique opportunity to better understand what causes these diseases, but also to develop new treatments, since it's been shown that drugs whose mechanisms of action are supported by genetics are much more likely to become approved therapies. But there's a problem – although genetic studies have successfully pinpointed the regions of the genome that are involved in autoimmune disease, we don't understand what actually goes wrong to bring about disease.

The use of genetically altered mice to study complex immunological processes (including both physiological and pathophysiological immune responses) has revolutionised the study of gene function by enabling almost every aspect of mammalian physiology to be interrogated in an in vivo setting.

Importantly, both murine immune responses and gene function are very similar to humans - a fact on which numerous insights into human immune function have been built. For example, mouse models of inflammatory arthritis and colitis have made it possible to study the processes involved in autoimmune disease development, while models of infection and cancer have enabled the function of the immune system to be studied in other important contexts. We propose to use these and other established mouse models to better understand the physiological functions of regions of DNA that directly contribute to the development of immune-mediated diseases or to the normal functioning of specific

immune cells. Importantly, because immune responses are also involved in fighting off infection and killing cancer cells, it will also be critical to ensure that targeting these pathways with drugs would be safe. This understanding may also provide insights that could even be used to improve treatment of cancer and infection. In summary, we aim to better understand how aberrant immune responses can lead to a range of different human diseases and use this knowledge to identify better treatments.

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

We anticipate several distinct outputs to have occurred by the end of this project:

New knowledge - we will gain better understanding of the biological processes that lead to autoimmune diseases, whether these processes could be targeted therapeutically, and whether they are also involved in responses to infection or cancer.

Publications - we will publish our results in high impact journals to communicate them to the broader scientific community, and ensure that the advances in knowledge that we generate can be widely utilised.

Intellectual property - we anticipate that the identification of pathogenic pathways and assessment of whether they could be targeted therapeutically will generate intellectual property, which will be owned by the host institution and used to ensure potential candidate therapies will be widely available.

Products - we anticipate that we should be able to identify small molecules that can modulate the pathogenic pathways we identify. We will partner with pharmaceutical companies to maximise the potential of any such drugs as therapies.

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

There are likely to be both short and longer-term beneficiaries from these outputs.

In the short-term, the discovery of previously uncharacterised disease pathways and how these are modulated by genetic variants will benefit the broader scientific community. Specifically, this will benefit

(1) complex disease geneticists with an interest in how disease-associated SNPs contribute to disease, (2) immunologists with an interest in biologically important processes involved in normal immune function, (3) clinicians who treat patients affected by these diseases, including autoimmunity and cancer, and (4) the pharmaceutical industry who are interested in finding new treatments for patients. Publishing our work will also benefit the researchers involved - improving their chances of obtaining independent funding for research and advancing their career progression.

In the longer term, the principal beneficiaries are likely to be patients affected by these diseases and their families, since we anticipate that these outputs will eventually lead to new treatments becoming available. This will also benefit clinicians, who will acquire a broader therapeutic armamentarium. In addition, there may also be wider socio-economic benefits - afforded by better disease control leading to lower sickness rates and a lesser need for intensive follow-up leading to reduced healthcare costs.

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

We will seek to maximise the outputs of this work at multiple stages within the research process.

During the conduct of the research we will actively collaborate with other research groups to both share our expertise and knowledge and to benefit from their expertise and knowledge. This will maximise our progress and ensure that we can operate efficiently - speeding up the work and ensuring we and others can generate reliable, reproducible data that affords genuine insights into disease biology.

After individual projects are completed, we will seek to disseminate the results to the research community as well as with potentially interested wider audiences. This will be achieved by presentations at local, national and international meetings (including seminars, conferences and workshops). In addition, presentations will be made at clinical meetings - both locally and nationally - in order to communicate results to specialist clinicians. Publications resulting from this work will be highlighted on social media and institutional and personal websites, in addition to writing press releases. We will also ensure that the results are communicated to the non-research community through presenting at public engagement events and to patient groups, disseminating lay summaries of our results / publications on social media, and working with the communications team at the host institution.

To maximise the utility of the results for the research community, we will make raw data available to other researchers by deposition into publicly accessible repositories after publication (e.g. genomics data will be deposited in NCBI GEO).

To maximise the chance of translating our research into clinically effective therapies, we will establish industry partnerships (e.g. with pharmaceutical companies) to help develop and further optimise any potential therapies.

To avoid other scientists having to perform similar experiments that we have already shown to be unsuccessful, we will ensure that important and definitive negative results are published, as well as positive results.

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.

Genetically modified mice have revolutionised biology by enabling almost every aspect of mammalian physiology to be investigated in an in vivo setting. For the proposed work, mice represent an excellent model, not only because genetically-modified strains can be rapidly made, but because their immune responses, gene functions and transcriptional regulation are very similar to humans. Indeed, we will only study disease-associated loci that share high sequence homology in mice and are syntenic, both in terms of conservation of order of local genes and enhancer activity. For most experiments, we will use mice between 6-10 weeks of age, at which point they have a fully developed immune

system. This is in accordance with established protocols for the disease models we will be using.

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

All mice will undergo a tissue biopsy from the ear to determine their genotype, and for a number of animals this will be the only intervention as further study would be on post-mortem immune cells. In a proportion of mice we will induce colitis, infection, arthritis, or tumours. Induction of these models requires the introduction of an initiating agent (e.g. DSS, emulsified collagen, bacteria or tumour cells). The route of delivery for these agents varies depending on the model, but is typically one of: oral (e.g. DSS colitis), intraperitoneal (e.g. T cell transfer colitis), subcutaneous (e.g. tumour models), intravenous (e.g. salmonella infection) or intradermal (e.g. collagen induced arthritis). The resulting disease can be associated with diarrhoea (e.g. colitis models), joint swelling (e.g. arthritis models), weight loss or compromised overall well-being (all models). These experiments will vary in length, from 2 weeks for some colitis models (e.g. DSS colitis) up to 10 weeks for collagen-induced arthritis.

Animals will be monitored on a regular basis to detect any sign of distress or suffering. The majority of animals studied in this project licence will experience subthreshold or mild severity. The proportion of animals that experience moderate severity symptoms / signs will vary between models (the anticipated numbers are indicated in each protocol) but we will aim to minimise suffering using experimental refinements (e.g. pre-emptive analgesia) and less severe models wherever possible. Any animal that reaches the humane endpoints will be killed. In the event of complications, or at the end of the experiment, animals will be humanely killed by a schedule 1 method.

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

The vast majority of genetically-altered mouse lines we study develop entirely normally and none should experience any adverse events from the tissue biopsy required for genotyping. Mice included in colitis or arthritis experiments are expected to develop diarrhoea or joint swelling respectively, which
may be associated with weight loss, discomfort and compromised overall well-being. Mice included in infection experiments will develop signs of the infection. Mice included in tumour experiments may experience mild discomfort at the site of the tumour and compromised overall well-being.

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

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

Embryo recipients: Expected severity = MILD (50%); Highest severity that can be experienced = MODERATE (50%)

Long-term maintenance and phenotyping: Expected severity = MILD (25%); Highest severity that can be experienced = MODERATE (75%)

Colitis models: Expected severity = MODERATE (100%); Highest severity that can be

experienced = MODERATE (100%)

Arthritis models: Expected severity = MODERATE (100%); Highest severity that can be experienced = MODERATE (100%)
Infection models: Expected severity = MILD (20%); Highest severity that can be experienced = MODERATE (80%)

Tumour models: Expected severity = MILD (20%); Highest severity that can be experienced = MODERATE (80%)
Pharmacodynamic protocol: Expected severity = MILD (40%); Highest severity that can be experienced = MODERATE (60%)

For all other procedures, 100% of mice are expected to have mild severity only.

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 autoimmune and inflammatory diseases we are studying - ranging from inflammatory bowel disease (IBD) to rheumatoid arthritis - are complex immunological disorders whose biology cannot be fully recapitulated in vitro. For example, IBD involves interactions of multiple components of the host immune system with the intestinal epithelial barrier, mucus layer, and trillions of viruses, fungi and bacteria in the gut – all of which needs to be triggered by poorly understood environmental factors and to occur in genetically susceptible individuals. While we extensively use in vitro human studies to delineate several aspects of how genetic variants affect immune cell function, there are no in vitro models that can fully capture the complexity of these diseases – especially if the genetic loci being studied are biologically active in a broad range of different cell types. We therefore need to use mouse models to discover how candidate genes / loci affect physiological and pathophysiological immune responses, including the development of organ-specific autoimmunity and determine whether these pathways also affect immune responses to infection and cancer.

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

We use an extensive range of in vitro human cell culture (and co-culture) systems and in silico systems biology methods (e.g. pathway enrichment analysis, co-expression and interaction analyses, and network analysis) in our work. These include assays of immune cell function as well as methods to characterise the consequences of modulating putative disease pathways. These are full reduction methods that will allow us to make predictions regarding the likely effects of specific genes / loci and then prioritise those mechanisms that need to be studied in mice in vivo. Similarly, candidate drugs will be identified from small molecule screens using human immune cells in vitro (e.g. T cells, NK cells and macrophages) meaning that focused in vivo experiments can then be performed, removing

the need for in vivo screening. Ex vivo experiments using samples obtained following a humane method of killing will also be used - where possible - to confirm biological mechanisms without the need for additional regulated scientific procedures.

Why were they not suitable?

These methods are suitable for the initial phases of this work and for certain validation experiments and will collectively help us reduce the number of animals that would otherwise be required. However, the complexity of immune-mediated disease - including the involvement of rare cell types that may not be obtainable in sufficient numbers (if at all) from human tissue - means that an in vivo approach is necessary to fully understand the role of certain genes / loci in the development and subsequent course of disease.

Reduction

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

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

We consistently aim to minimise the number of animals we use per experiment - consistent with the reduction principle of the 3Rs. This is achieved by ensuring that the experiments are well-designed, appropriately controlled and correctly analysed, and collectively lead to a reduction in animal use while maintaining the reliability and reproducibility of the results.

In terms of study design, we use the Experimental Design Assistant - a free resource provided by the NC3Rs - to ensure that an appropriate number of animals are used for each experiment. In addition to providing support for power calculations to determine the required sample size, this also helps ensure that appropriate randomisation procedures are used when allocating animals into particular study groups (e.g. treatment with a drug or a vehicle control) and that lab members remain blinded to genotype / treatment allocation when performing the experiment, assessing outcomes, and collecting the data. For every experiment we perform, we always first consider the size of the effect that we would like to be able to detect. We specifically focus on being well-powered to find physiologically relevant (large) effects between treatment groups (e.g. drug vs control or knockout mice vs wildtype littermates). To do this, we typically use a standardised effect size of 1.5, which is likely to be biologically significant - per https://eda.nc3rs.org.uk/experimental-design-group#usingcohensd. We then estimate the expected variability between mice in the experimental model, either using our previous experience of these models or from the literature / collaborators or by performing small pilot studies. These data are then used to inform our sample size calculations, which also consider the statistical significance threshold (alpha), required power (1 - beta), the expected distribution of the data, and whether one- or two-tailed tests will be used.

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

We design our experiments rigorously to ensure that they are statistically well-powered to

detect a biologically significant effect of the size that we expect based on prior knowledge. We carefully consider which experimental design would enable us to answer our scientific questions using the minimum number of animals (including factorial designs for assessing different levels of a variable in the same experiment and block randomisation to control confounding factors and minimise variability). This means that we use the minimum number of animals to ensure that our scientific objectives are met. We ensure that animals in experimental groups are matched for age and sex using littermates to control for effects that relate to the composition of the study groups rather than the variable being tested. This is important as both age and sex are known to affect immune responses. Using littermates will also control for any effects of the gut microbiota which can also affect the immune system. We also consider what the most appropriate animal characteristics are for each experiment. For example, mice of certain ages or strains may be more or less susceptible in certain experimental models.

Ensuring that we use animals with the most appropriate characteristics for an experiment improves the applicability and robustness of the results. We additionally consider whether there are other independent variables that might affect the results of an experiment, but which are of no interest in themselves. These are termed "Nuisance variables" in the EDA and are important to control since they can both confound results (if they are imbalanced between experimental groups) or increase the variability in the outcome measure, thereby reducing experimental power. To minimise bias, we commonly use block randomisation to control for relevant animal characteristics and nuisance variables in experiments. Where necessary (if we do not have an understanding of the variability expected in an experiment) we perform small pilot studies to inform our subsequent power calculations - thereby ensuring that we do not waste animals by conducting a study that is unnecessarily large. We also consider in advance the nature of the data that we expect to collect (e.g. continuous vs categorical, parametric vs non-parametric) and formulate an analysis plan to ensure that the statistical test we will use - to compare the results against the null hypothesis - is the most appropriate. Finally, we always consider the potential scope to maximise the data gained from every mouse. For example, taking samples for unrelated ex vivo experiments after killing by a schedule 1 method at the end of an experiment.

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

In addition to good experimental design, we also use general reduction methods to optimise the number of animals we plan to use. If we plan to generate a new genetically-modified mouse line, we first search a broad range to databases (e.g. www.knockoutmouse.org, www.informatics.jax.org, www.mousephenotype.org etc) to determine whether such mice might already exist and thus be available without the need to produce a new line. For example, during our previous work we wanted to obtain a knockout mouse for a gene we were studying and ended up sourcing a suitable line via a group in Australia rather than having to make a new line. In addition, we also ensure that all of our genetically-modified mouse lines are cryopreserved (either as frozen embryos or sperm) to minimise the number of lines that are actively maintained at any time (and also ensure that we are in a position to share lines with other groups upon request). Our mouse colony is managed by an experienced Senior Laboratory Research Scientist with expertise in careful colony management to ensure that we only breed mice that we need for experiments or maintenance of a line. Similarly, we ensure that the strain and substrain of any wild-type mice that we intend to breed with genetically-modified mice is suitable to maintain the genetic background of the colony. This way, we avoid changes in phenotype resulting from alterations in the background strain of the mice.

Refinement

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

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

All of the experiments proposed in this project will use mice. The specific models and methods that we intend to use do not typically require animals to go through more than mild suffering, and have previously been refined to minimise any distress. These models are as follows:

Colitis models (DSS, Hapten, T cell transfer, spontaneous colitis due to genetic modification)

Arthritis models (collagen-induced arthritis, anti-collagen antibody-induced arthritis, K/BxN serum transfer model)

Immunological challenge models (including primary and secondary immunisations with or without adjuvant containing vaccines)

Infection models (Influenza, MCMV, LCMV, Salmonella, Helicobacter, Citrobacter, Mycobacteria, Listeria)

Tumour models (subcutaneous implantation of tumour cells)

All of these models may incorporate steps to help understand the biological processes involved, including irradiation and bone marrow transplantation, administration of drugs / cells (may be repeated), general phenotyping including withdrawal of blood from a superficial vessel. All of these methods have been refined to minimise the suffering to the animals. For example, blood withdrawal will not exceed 10% of total blood volume at any time, so as not to adversely affect the physiology of the animals.

In addition to these experiments, we will also use general breeding and maintenance methods for GA mice, superovulation, and embryo transfer.

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

A major advantage of using mice for the proposed models and experiments is that their immune system, its regulation, and the specific genes that control this, are very similar to humans. Maturation of the mouse immune system is complete when mice enter adulthood (approximately 6 weeks) and hence it is not possible to use younger animals or embryos for these models. Similarly, invertebrates (e.g. Drosophila or C.elegans) are unsuitable for these models due to major differences in their immune systems and gene function. Models of the diseases we will be studying (and which we propose to use) have already been

established in mice and are in widespread use in research.

Equivalent models - that recapitulate many of the features of human disease - do not exist for less sentient animals.

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

We will continually review our experimental protocols to determine whether there are any improvements that we can make in experimental design, techniques and animal husbandry to improve welfare and minimise adverse effects. This will involve both reviewing our own performance and also applying external knowledge / advances in best practice. As part of this, we will also maintain and share detailed information on the experimental procedures we use to benefit other groups. We will perform increased monitoring during all experiments to be able to detect any adverse outcomes and if we are performing a new procedure or an established procedure on a new strain we will undertake a pilot study to establish optimal experimental conditions (that minimise suffering) and confirm the appropriate humane endpoints. We will work together with animal care staff to ensure that animals are maintained in the most appropriate environment, with adaptations being implemented for certain models (for example, for arthritis models we will provide softer bedding and ensure that food is also provided on the floor of the IVCs to ensure that animals with arthritis are able to feed normally). We will use anaesthesia and analgesia whenever appropriate (including pre-emptively) and will perform post- mortem examinations to assess any unexpected deaths. We already have a track record of refining experimental procedures to minimise adverse effects - for example, we adapted the collagen-induced arthritis protocol to reduce the volume and number of emulsified collagen injections, and showed that this reduced the incidence of dermal ulcers.

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

Prior to commencing any experiment, we will review a series of resources to ensure that our experiments are conducted in the most refined way. This will include reviewing guidance produced by NC3Rs expert working groups on specific disease models or more general guidance on common procedures, such as blood sampling (available via the NC3Rs website) and publications from the RSPCA Science Group regarding refinements to specific procedures and models. We will also use other sources, including the scientific literature (e.g. Nature Protocols / JOVE) and guidance available from companies such as Jackson laboratories, who have produced a series of manuals and guides for specific experimental models (see https://www.jax.org/jax-mice-and-services/customer-support/manuals-posters-and-guides/jmcrs-manuals-guides). We will also discuss with the NACWOs and technicians at the Biological Research Facility to determine whether they know of any recent developments in experimental protocols that could be used to further refine our experiments.

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

I have subscribed to the monthly NC3R e-newsletter, which will help me stay up to date with advances in the 3Rs and how these can be implemented. In addition, I plan to have regular discussions with the Named Persons and animal technicians at the establishment

to review our approaches and determine whether there are any new opportunities to implement advances in the 3Rs. I can also contact the local NC3Rs Regional Programme Manager for specific advice as necessary. I am also aware that NC3Rs events and workshops are regularly posted on the NC3Rs website (events page). I will use this to identify any suitable opportunities to stay informed of 3Rs advances and new approaches.

6. The multi-system impact of altered feeding patterns

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Feeding patterns, Endocrinology, Metabolism, Brain health, Prader-Willi syndrome

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

This project will determine the impact of temporal feeding patterns on endocrine, metabolic and neurogenesis-related endpoints and the mediatory role of the stomach hormone ghrelin in these responses. In addition, this project will characterise the impact of the loss of genes from the Prader- Willi locus on the development of neuronal morphology and function, and related behavioural indices, together with the influence of ambient temperature on on physiological endpoints in pattern-fed and Prader-Willi syndrome mice.

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

Why is it important to undertake this work?

It is clear that human feeding behaviour has altered significantly over the last century. While much research has focused on the consequences of altering dietary content, the impact of altering the temporal pattern of feeding patterns on health remains a major unanswered question. Epidemiological studies have suggested that altering the timing,

duration and frequency of feeding may influence a range of physiological and pathophysiological processes, including energy consumption, fat storage and cancer susceptibility. However, these studies are compromised by their reliance upon self-reported food intake and participant attrition. While we are working with clinical collaborators to improve the robustness of these human studies, detailed understanding of the physiological impact of altered feeding patterns and the mechanisms underpinning these effects can only be achieved in the context of in vivo studies performed rodents in the tightly regulated environment of the laboratory.

Given that the the secretion of the hunger hormone, ghrelin, undergoes dynamic fluctuations in response to feeding and that the biological activity of this hormone is dependent upon the pattern of exposure, much of our work to determine potential biological mechanisms will be centred around this hormone and its receptor.

In addtion, Prader-Willi syndrome is an anherited condition usually associated with chronic over-eating and obesity. However, one aspect of this condition that has received little scientific attention is the problematic alteration in cognitive performance. We will therefore characterise the impact of the loss of Prader-Willi syndrome genes on neuronal development and its consequences for neuronal function and related behaviour in mice. Since this condition is also associated with elevated circulating ghrelin, we will explore the potential role of this hormone in mediating these effects.

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

In the short-term (within the timeframe of this project), this work will lead to the publication of new information on:

The impact of feeding patterns (including grazing, meal-feeding and intermittent fasting) on a range of physiological endpoints including:

Caloric intake and dietary selection

Hormone secretion, growth and metabolic outcomes

The formation of new nerve cells and related behaviours (spatial learning and anxiety)

The role of hormone systems (including ghrelin) in mediating these effects

This work addresses a major unanswered question in nutritional science and will include establishing the difference in responsiveness between males and females

The impact of the loss of genes from the Prader-Willi syndrome (a condition of chronic over-eating) locus on neuronal development, neuronal function and related behaviours

The effectiveness of novel compounds designed to reduce over-eating in Prader-Willi syndrome

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

In the short-term (within the timeframe of this project), this work will:

Inform our collaborators running parallel studies in human volunteers about potential new

endpoints and mechanisms for investigation
Establish the impact of the loss of Prader-Willi syndrome genes on neuronal development and related cognitive performance

In the medium to long term (beyond the timeframe of this licence), this work should:

Provide reliable scientific evidence about the most healthy feeding patterns for domestic, captive and laboratory animals

Predict whether cost-neutral manipulations in feeding patterns represent a useful non-therapeutic strategy in combatting obesity in humans

Predict whether cost-neutral manipulations in feeding pattern will be a useful strategy in promoting healthy growth in regions of the developing world where nutrient availability is limited

Develop our understanding of the wider physiological consequences of using food restriction to enhance motivation in behavioural testing paradigms laboratory animals

Predict whether cost-neutral manipulations in feeding pattern or activation of ghrelin signalling impact brain development and health

Determine the cellular basis of disrupted cognitive development in children with Prader-Willi syndrome

Provide the basis for seeking new therapeutic strategies for improving cognitive performance in human Prader-Willi syndrome and establish the potential benefit of novel therapeutic agents in alleviating the over-eating associated with this condition

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

In order to maximise the outputs from this project we will:

Continue to develop our emerging collaboration with colleagues running parallel studies in human volunteers

Present our findings in premier scientific conferences

Publish our findings in high-impact scientific journals

Present our findings to wider audiences by engaging with the popular media and utilisation of online platforms

This research will produce reliable empirical evidence to inform parents, clinicians, dieticians, educators and policy makers about the impact of specific human feeding patterns on a range of metabolic, developmental and behavioural endpoints, and could have profound implications for determining the most healthy way to feed our children.

Species and numbers of animals expected to be used

- Mice: 3000
- 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.

We will use rats in our studies where appropriate models are available. Rats are the species of choice when processes such as hormone secretion are being monitored dynamically. This is not feasible in mice as they are too small to be subjected to the automated serial blood sampling necessary for the collection of multiple blood samples and subsequent dynamic profiling. Rats are also the species of choice when assessing dietary selection as most mouse strains show an almost exclusive preference for calorie-dense diets that is difficult to manipulate.

We will use mice in our studies where appropriate models are available. Mice are the usual species of choice because the range of genetically-altered models available is large and varied. This enables combinations of models to be used to address very specific mechanistic questions (e.g. what is the role of a particular hormone receptor in this tissue/cell type) to be addressed. These are very powerful and informative approaches to discovering potentially important druggable targets for human application.

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

The most commonly used protocol will involve dietary variation (Protocol 3). Under this protocol, rats or mice (some with genetic alteration) will typically experience injection of a compound to label new- born cells, followed by single housing and the supply of diet in a regulated pattern (e.g. nocturnal grazing or meal-feeding, or exposure to intermittent fasting (such as the 5:2 diet, where animals experience 2 non-consecutive dayas of fasting per week and 5 days of ad libitum-feeding)) for periods of up to 6 weeks. This may also co-incide with increasing or decreasing the ambient temperture to either suppress or activate thermogenesis.

During the last week of the feeding pattern period animals may undergo a series of behavioural tests (e.g. dietary selection, spatial memory or anxiety testing). This may involve several days of acclimatization to an arena or maze, prior to an object recognition/location analysis (usually a 30 min period for each animal).

Alternatively, rats may be surgically prepared with indwelling vascular catheters during the last week of feeding followed by automated serial blood sampling to assess the impact of feeding patterns on dynamic hormone profiles.

Pattern-fed rodents will typically be killed by decapitation under terminal anaesthesia, to enable collection of trunk blood and other tissues to assess the effects of these feeding patterns on a range of biological end points (e.g. skeletal growth, adiposity and new nerve cell formation).

Genetically-altered mice (e.g. bearing mutations in the Prader-Willi locus) will be bred and experience a battery of behavioural tests to assess cognitive performance prior to ex-vivo analysis of neuronal function or post-mortem quantification of neuronal morphology.

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

The majority of procedures are not expected to cause any adverse effects.
Singly housed animals may experience mild isolation stress, but this is usually only noticeable using complex assessment of anxiety-like behaviour. Animals will only be singly housed for periods of up to six weeks.

Surgical procedures are expected to result in pain, but this will be mitigated by being performed under anaesthesia couple with the routine use of analgesia. Animals prepared with vascular catheters will not typically be maintained beyond 14 days after surgery.

Most other procedures are not expected to cause anything more than transient distress or pain (e.g. administration of substances) and these will typically not be repeated on more than four consequtive days. or negligible pain, distress or lasting harm (e.g. dietary variation or behaviour testing).

Mice bearing deletions or mutations in genes within the Prader-Willi locus will typically show mild reductions in skeletal growth, reduced weight gain (fat mass) and elevated food intake in proportion to their body weight. Mice with loss of multiple PWS genes (e.g. mice with mutations of the imprinting centre) display increased neonatal mortality, with surviving offspring showing more profound reductions in weight gain.

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

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

The majority of protocols in this project will not exceed a mild severity limit, only those involving surgery (<10%) and genetically-altered mouse models of "full" Prader-Willi syndrome (<5%) may be considered moderate. Some procedures (e.g. the breeding of genetically-altered rodents) are not expected to induce any pain, distress or lasting harm and, in the absence of any subsequent procedures may be considered sub-threshold (<20%).

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 study of the physiology of feeding patterns is a relatively new scientific discipline.While it is possible to expose cultured cells to nutrients in user-defined temporal patterns, it is emerging from our previous studies that feeding patterns influence a wide range of physiological endpoints, including adiposity, growth, fertility and

neurodegeneration. Given that these systems also interact with each other, the complex holistic impact of temporal feeding patterns can only be meaningfully studied in the context of the whole organism.

Thus, whilst we are continuing to use non-sentient alternatives to study individual components of this system, e.g. to:

Quantify the direct actions of ghrelin on apoptosis and neurogenesis in cultured hippocampal neurones

Quantify the direct impact of loss of specific PWS genes on the morphological development of cultured cortical neurones

the overall impact can only be satisfactorily assessed in vivo.

Epidemiological studies to address this developing area of science have been compromised by reliance upon the self-reporting of food consumption (which is widely recognized as inherently unreliable). While we are beginning to collaborate with colleagues conducting intervention studies in human volunteers, this needs to be approached with caution, since it is possible that temporary changes in feeding patterns may induce irreversible changes in subsequent feeding behaviour and consequent changes in metabolism. In this context, it is necessary to perform controlled feeding studies in laboratory animals. This approach has the additional advantage that use of genetically- modified animals (e.g. with specific gene deletions) will enable us to establish the molecular mechanisms underpinning the observations we are making by assessing the contribution of specific gene products to the effects of patterned feeding.

Similarly, the study of mouse models for Prader-Willi syndrome presents an exciting opportunity to understand the mechanism linking loss of these specific genes from the PWS locus and impairment of cognitive performance and cellular morphology. This mechanistic information, unobtainable in humans, will produce evidence invaluable in the treatment and management of humans with PWS.

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

In collaboration with our colleagues we have used/are using:

Cultured hippocampal neurones to quantify the direct actions of ghrelin on apoptosis and neurogenesis

Cultured cortical neurones to quantify the direct impact of loss of specific PWS genes on morphological development

Why were they not suitable?

While it is possible to expose cultured cells to nutrients in user-defined temporal patterns, it is emerging from our previous studies that feeding patterns influence a wide range of physiological endpoints, including adiposity, growth, fertility and neurodegeneration. Given that these systems also interact with each other, the complex holistic impact of temporal feeding patterns can only be meaningfully studied in the context of the whole organism.

Similarly, while cultured neurones enable definition of the direct impact of loss of specific

PWS genes on neuronal development, the environment created by neighbouring non-neuronal cells also impinges on morphological development. In addition, these morphological impairments will lead to alterations in cognitive performance. Since this is poorly understood in PWS, the use of in vivo models is essential to understand how local cellular processes lead to holistic delveopment and behavioural performance.

Reduction

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

Considerable past experience (>25 years) in running these types of studies

Sample sizes of 6-7 for endocrine and metabolic studies and 10-12 animals/group for behavioural studies respectively

Current usage rates (approximately 200 mice and 50 rats pa)

An assumption that our research group will continue to grow at the same rate as in the last three years.

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

The number of animals used are minimized by:

Developing a library of biological materials and analysing multiple systems in single experiments. For example,

Temporal feeding pattern studies routinely end with the collection of plasma, brain, pituitary, white adipose tissue (inguinal, gonadal, retroperitoneal), brown adipose tissue (interscapular), adrenal glands, liver, kidney, femur and tibiae, with optional collection of stomach, small intestine, colon, spleen and heart.

Studies with PWS mouse models are currently focused on collection of brains, but we routinely collect plasma, pituitary, femur and tibia.

Thus, repeating individual manipulations with separate cohorts of animals is usually unnecessary as we have sufficient material for analysis to answer most questions that arise. The success of the strategy is seen from the publication of multiple papers from the same animals. For example, our analysis of skeletal performance in PWS mice was largely achieved by analysing bone samples collected from the study of metabolism in the same mice, with pituitary samples also contributing to an analysis of pituitary sensitivity .

Quantifying multiple hormones in single samples (e.g. GH and corticosterone).

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

The number of animals used in our studies are optimised by:

Maintaining small breeding colonies of our genetically-altered mice, breeding only those mice sufficient for maintaining the colonies and the supply of planned experimental cohorts. Animals not used in either of these ways are utilised in pilot studies to determine the value of potential new directions within the remit of the licence.

Only establishing colonies of mice when the shipping of tissue is not a viable option.

Sharing samples from our tissue library with national and international colleagues (see comments above).

Refinement

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

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

Models:

Rats are the most appropriate species for hormone/nutrient profiling because they are large enough for the application of serial blood sampling protocols required to assess dynamic profiles.

Rats or mice will be used where appropriate genetically-modified strains are available or can be generated de novo (outside the scope of this licence) for either the recapitulation of novel models of human diseases (e.g. Prader-Willi syndrome) or for experimental approaches to establish physiological/pathophysiological mechanisms (eg. the absence of ghrelin, or its receptor). In this context novel PWS rat models offer the advantage that automated blood sampling can be used to establish the exact hypothalamic mechanisms underlying the impairment of hormone secretion.

Methods:

The three most commonly used methods to be employed under the authority of this project are:

Automated feeding: We use Columbus Instruments' comprehensive laboratory animal monitoring system (CLAMS)-based feeding station as it represents the most advanced system for regulating the pattern of food access. By using crushed diet instead of pelleted diet, this system avoids the complications arising from food hoarding. To overcome the

potential detrimental effects of single housing, feeding cages are in close proximity on the station, enabling visual and auditory contact. We have developed additional environmental enrichment for routine use in this system, including back wall scenes, half-ball housing and ball swings. These bespoke developments enrich the environment without interfering with the feeding equipment, indwelling catheters or behavioural end points.

Automated blood sampling: This is required for the quantification of circulating hormone levels where secretion is characterised by frequent spontaneous bursts and/or daily rhythms. This equipment is the most advanced system designed for the collection of serial blood samples, featuring a number of refinements, for the benefit and safety of the animals.

Behavioural analysis: The range of tests selected to assess the impact of altered feeding and hormone patterns and loss of PWS genes on behaviour represent those that cause the least pain, suffering, distress or lasting harm. These include optimized methods assessing spatial memory, emotional reactivity and food preference.

Other techniques include:
Oestrus testing: The stage of reproductive cycle will be determined by the simple swab technique.

Implantation of delivery devices: These will be used to deliver nutrients, hormones or compounds.

Acute cannulation: This approach will be used where prolonged compound delivery and/or blood sampling is not required and where the analyte to be measured is not affected by the presence of anaesthesia.

Ambient temperature modification: Modification of ambient temperature will be used to suppress or enhance thermogenesis.

The techniques used represent the most refined approaches available, with the project formulated to exploit and advance these refinements. Where new to my laboratory, pilot experiments will establish the minimum time points necessary to provide reproducible results for each protocol, whilst causing the least pain, suffering, distress or lasting harm.

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

Many of the studies in this project employ long-term regulation of feeding patterns or hormone treatment, with assessment of behavioural outcomes. While less sentient alternatives may be appropriate for more acute, mechanistic studies, they either do not have the same complex of interacting physiological processes or are inappropriate for the measurement of complex behavioural outputs (for example anxiety and spatial memory).

Terminally anaesthetised animals can only be used for studies not requiring spontaneous feeding behaviour or other behavioural outputs, or studies quantifying hormone secretion (anaesthesia suppresses the secretion of hormones controlling skeletal growth).

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

Breeding: Offspring will be identified by appropriate husbandry methods, which cause no more than momentary discomfort. (eg. ear marking, or the implantation of microchips), the mildest appropriate method being used for positive DNA genotyping.
Due to the higher than average mortality rate of PWS-IC pups, animals bred under this protocol (Protocol 06) will be subjected to additional condition scoring (eg. the system of Wolfensohn S & Lloyd M in Handbook of laboratory animal: management and welfare, Blackwell Publishing 3rd edition, pp59-73 (2003)), those pups with a poor score (9-12 using the above system) and therefore unlikely to survive, being killed.

Feeding: While our feeding station represents the most advanced system for regulating the pattern of food access, and incorporates a number of important bespoke modifications to enhance the environment, ongoing discussions with the manufacturers improving/enhancing the technique and reducing the impact on the animals studied.

Environmental enrichment will always be used where it does not interfere with feeding equipment, indwelling catheters or behavioural end points. This will be assessed on a case-by-case basis, after consultation with the NACWO.

Temperature: Since thermoneutrality in mice is 30-31°C, thermogenic stress may be reduced by maintaining mice at non-code of practice temperatures at or just below the thermoneutral zone (30°C).

Surgical procedures: In all surgical procedures the following refinements will be employed to ensure the least pain, suffering, distress and lasting harm.

Anaesthesia: The most appropriate inhalation or injectable anaesthesia will be used.

Analgesia: Analgesics will normally be administered prior to surgery, with additional analgesia administered at the end of surgery if necessary. Timing and treatment may be modified if standard practice is predicted to have a confounding influence on the end results (eg. feeding behaviour or hormone secretion).

Surgery: Aseptic technique will be used.

Automated serial blood sampling: While our system represents the most advanced approach to serial blood sampling, incorporating a range of novel bespoke modifications, further alterations to the software, and operation of the system will be incorporated to minimise the risk of causing pain, distress and lasting harm.

Behavioural analysis: To minimise pain, distress or lasting harm, our behaviour testing protocols will incorporate elements of environmental acclimatisation and, where appropriate, training.

Prader-Willi Syndrome models: Genetically altered animals that already exist and may be used under this project have no phenotype or only a mild phenotype except for the IC-deletion mouse model of Prader-Willi syndrome. These animals have an overt harmful phenotype particularly in the neonates. The severity will be minimised through the use of breeding strategies to avoid production of homozygous animals and enhance maternal behaviour, while a monitoring scheme will be used to identify suitable end points. Where appropriate, alternative, mild severity, models will be used where the endpoint under investigation is observable.

No severe procedures will be employed.

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

Overall, our protocols will conform to the best practice ARRIVE guidelines. In addition, our membership of specialist groupings publishing global online information keeps us abreast of advances in our specialist areas.

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

We will remain informed about advances in the 3Rs as they relate to this project by:

Attending national and international scientific meetings at which advances in methodology are presented

Maintaining membership of online fora for:

Automated serial blood sampling: Membership of the Pre-clinical Infusion and Sampling Technology online forum, permits discussion and adoption of the most recent advances in this technology, enabling advice to be given to/gained from other licence holders.

Touchscreen assessment of behaviour: Membership of an online touchscreen users group provides a quarterly newsletter in which advances (e.g. in different approaches to motivation, novel tests for pattern recognition) and best practice are presented and discussed. Similar information has been gained by attendance at the International Touchscreen symposium.

7. Preclinical models to study cancer biology and new therapeutic strategies

Project duration

5 years 0 months

Project purpose

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

Key words

Cancer, Genetic mouse models, Therapy, Tetastasis, tumour evolution

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim is to carry out fundamental cancer biology and translational research to deepen our understanding of the processes that underlie common cancers such as colorectal and pancreatic cancer, and to use the most relevant mouse models of cancer to investigate new and improved therapies that will benefit patients.

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

Why is it important to undertake this work?

Cancer is a major health issue for the population and accounts for more than one quarter of all deaths in the UK while being a significant burden on the NHS. We need advanced models to study the complex interaction of cancer cells in their natural microenvironment and which accurately represent the human disease as a means of identifying new

treatment options for patients.

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

Projects covered by this licence will lead to new knowledge in fundamental cancer research and increase our understanding of how tumours initiate, grow and spread to other organs. In particular, we will tease apart how cancers are influenced by their environment and metabolic dependencies.

Our goal is that these projects will identify new pathways for therapeutic intervention and lead to the development of novel anti-cancer therapies.

Work arising from these studies will be published in peer review journals and presented at national and international meetings to disseminate knowledge (to scientists and clinicians). We will also publicise our results to the public at open evenings, social media, and on our websites.

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

The knowledge gained from these studies will be of interest to the scientific community (short term) and cancer clinicians. Ultimately, we hope this will lead to new therapeutic approaches benefiting cancer patients (medium to long term). Furthermore, studies and validation in our preclinical mouse models should inform clinical trial design (medium to long term).

Ultimately, once we have developed improved cancer models that are more accurate and patient- relevant through our studies here, our goal is to establish a preclinical testing platform and through engagement with Pharma/small biotech/academic collaborators, which will improve accessibility for community investigators, as well as the pharmaceutical industry, to develop strong preclinical data and expedite development of new compounds of interest.

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

Our institutions will partner to centralise resources and expertise, making them available to the wider scientific community. We will also continue to collaborate with national and international colleagues and disseminate this work through publication and social media at the earliest opportunity.

Species and numbers of animals expected to be used

- Mice: 11,000

Predicted harms

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

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

The mouse is a mammal and warm-blooded which shares many features of human physiology and metabolism not found in other cold-blooded species such as flies and

worms. Genetic models of naturally occurring disease are currently the only way to accurately recreate the complexity of human cancers in a model system. Cancer is a disease that results from gene alterations (mutations and loss of normal genes) and therefore the best way to model the causes of cancer is to use genetically altered animals (GAAs) in which the same gene changes are recapitulated in the mouse. These GAAs are predisposed to developing cancer and act as a tool for us to test the principle of novel therapeutic strategies that could be used for patient benefit. We also use transplantation models whereby we can transplant tumour cells into specific sites of the mouse (e.g. colorectal cancer cells into the epithelium of the colon) to understand how the tumour, the host environment and therapeutic agents impact upon tumour progression. Cancer is an age-related disease and sometimes we have to age our cancer- prone mouse models (for up to 2 years) to mimic the clinical situation.

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

Animals with different gene alterations will be bred by conventional methods to achieve test subjects which may be predisposed to cancer. Approximately 70% of the mice will not show any adverse effects relating to their breeding and not undergo any procedures except for ear notching for identification and genetic testing. These will be humanely killed when they are no longer required for breeding. It is unfortunately necessary to breed so many animals to create the right combination of patient relevant gene changes in a few key cohort animals. In the future we hope to be able to circumvent the need to use so many animals but at the moment this is the best way for us to study the causes of cancer.

A proportion of animals (no more than 25%) will develop cancer because of their genetic makeup or because tumour cells have been implanted and allowed to grow. Tumour cells may be implanted into some tissues (such as the intestines) using a surgical procedure with appropriate anaesthesia and analgesia. Administration of an inducing agent to switch on/off particular genes may be done which only causes momentary discomfort but reduces off-target effects in other tissues.

Animals will be monitored closely by highly trained staff for well-established clinical signs such as weight loss, swelling of the abdomen, and development of palpable tumours. Some of these animals (15-20%) will be given anti-cancer treatments, changes in their diet, antibodies to reduce certain immune cell types, or agents to modify metabolism, and the response to these treatments monitored. All animals on treatment will be closely monitored and may be blood sampled to follow changes in biomarkers which should cause only mild handling stress and momentary discomfort or may be imaged e.g. by ultrasound or by rectal endoscopy under general anaesthesia. Any animal that displays signs of illness such as weight loss of normally 15%, but rarely up to 20%, immobility or ruffling of the coat will be humanely killed - this depends on the type of cancer but usually within 12 months and never longer than 24 months. At the end of the study all animals will be humanely killed and tissues collected at post-mortem to gather as much information from the study as possible.

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

Animals will be predisposed to tumour development. We are very familiar with the clinical signs (e.g. body condition, palpable tumour, paling of feet) associated with these tumours and all researchers and technicians are trained in these models. Any animal exhibiting clinical signs of cancer will be humanely culled at pre-determined end-points of no more

than moderate severity.

Anti-cancer treatments can cause gastrointestinal and haematopoietic disturbances as they do in cancer patients. Animals are monitored closely when undergoing such treatments and treatments stopped or animals killed if these effects cause suffering to the animal that cannot be resolved.

Surgical techniques are controlled with anaesthesia and analgesia and any animal who does not fully recover from such a procedure 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 of the animals on this project (70%) will have a subthreshold severity meaning they will show no harm.

About a quarter of animals (25%) will develop cancer in which 10% will have mild symptoms such as small palpable tumours or taken at a timepoint prior to clinical signs manifestation. The remaining 15% will exhibit moderate clinical signs associated to their cancer burden.

With respect to other procedural burden (e.g. imaging, anti-cancer drug treatment), 20% of animals will experience one or more procedures which will never exceed a moderate severity and where we anticipate 5% will experience a mild procedural severity with 15% experiencing a moderate procedural 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?

Although many aspects of cancer research can be conducted using cell lines, the potential of a putative cancer-causing gene and the accumulation of secondary events necessary for a cancer to fully form often needs to be assessed within the context of the whole animal. Furthermore, non-animal alternatives cannot totally model the complexities of cancer development as it is well recognised that the immune system, energy requirements and the tumour micro-environment play an important role in disease progression. Whilst the mouse is still not perfect for modelling a human being, we know that we can get closer to patient relevance using a living mouse model alongside our lab assays.

Most cancer patients die not from their primary tumour but when it spreads to other organs and to model this in the most effective way requires a living organism. Finally, we know

that cancer cells respond differently in the lab to anti-cancer therapies as they do in the context of the animal and so testing the efficiency of such therapies requires a complete animal system.

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

Our mouse experiments are an extension of solid lab-based observations and we only progress using mice when sufficient rationale is obtained based on *in vitro* cell culture. Where possible, we use 3D organoid based systems, patient material and mathematical models alongside our mouse models.

Organoids and 3D models are excellent ways to study genetic changes in a cancer cell and how this might affect cell growth and cell survival. We are also developing better ways to assay how cells invade and spread (as a model for cancer metastasis). We use these systems to check selectivity and specificity of a new compound for example, which allows us then to prioritise the best candidates to take forward in a physiological system for validating tumour targeting effects.

Why were they not suitable?

Modelling cancer and how cancers spread to other organs is extremely difficult in anything other than a whole animal. The interactions between the different cell types that make up a cancer (tumour cell, immune cells, blood vessels) is difficult to do in a dish. Patient samples tell us about end stage tumours but to understand what is happening at early stage of disease requires model systems.

Reduction

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

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

Cancer is a disease in which several different gene changes occur and so we have to breed many mice in order to achieve the 'sweet-spot' of gene alterations in any one animal - which can be 4 genetic alterations (including tumour suppressor alleles requiring deletion of both alleles) in any one cohort animal which requires a coordinated breeding plan to achieve this. Numbers are calculated based on our prior experience using the same models (>12 years), published literature and advice of our in- house statistical experts where minimum numbers are used but which will ensure robust statistical significance.

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

When planning our experiments we use pilot studies in the first instance, to inform on how many numbers we require for statistical significance and use our previous experience in these experiments, the NC3Rs Experimental Design Assistant or other statistical websites, which inform us of our effect size in order to guarantee our studies are meaningful. At our institute we have a system in place whereby researchers complete an experimental

collaboration form in discussion with the project licence holder to ensure the experiment is scientifically justified, efficiently planned and within the licence remit before any experiments can take place.

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

We maximise our breeding strategies to generate the most effective breeders to create the genetics of interest for our studies and we use tumour transplant models where appropriate, which do not require breeding of genetically altered animals and thus use fewer animals in total per study.

We use inbred strains of mice which are nearly identical to each other resulting in less variability between animals and allow us to use fewer animals to achieve a statistically significant result.

To reduce numbers of mouse-based experiments we always perform studies using cell lines or 3D models so that only our strongest hypotheses are tested in 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.

We use mouse models with the same genetic changes that are known to cause human cancer – so accurately replicating the human disease. These genetic changes are specifically altered in the tissue of interest (e.g. intestine) so that unrelated effects in other tissues do not occur. To induce gene expression, agents are given orally or by injection into the peritoneal cavity, under the skin, or directly into the colon by fine scope under anaesthesia. All animals are monitored regularly for signs of normal behaviour and are humanely killed if they exhibit moderate adverse signs. Staff will be expertly trained in these clinical signs. Regular monitoring of mouse welfare allows us to complete studies at the earliest endpoint in which we observe a significant result to prevent unnecessary suffering resulting from high tumour burden.

Anti-cancer therapies and agents to modify the immune system are given as they are in patients (e.g. orally, by injection under the skin, or directly into the blood system). Ultrasound and endoscope imaging under anaesthesia allows monitoring of tumour development over time before tumours cause clinical signs.

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

Cancer is a disease of ageing and is best studied in a mammalian system where many features of human physiology, the immune system and metabolism is recapitulated, such as in the mouse. This is especially important in the context of the tumour environment

which is critical in driving cancer, and where such cellular neighbourhoods are not accurately represented in cold-blooded species such as flies and worms. Where possible we do use less sentient species in aspects of our studies. For example, we use flies for colorectal research to study basic genetics, stem-cell generation and early stages of disease. However, this system has limitations as although the fly has an intestine it does not have a colon. It is also less easy to study later stages in cancer progression such as with metastasis, which is what kills most of the patients with cancer. Some therapeutic analysis can be studied in tissue-derived organoids (direct from human tissue or mouse-derived tumours) and in flies, but the full affect of drugs often requires the whole mammalian system.

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

Animals are housed in a dedicated facility proactive with environmental enrichment and receive anaesthesia and analgesia as appropriate.

All animals are welfare checked daily in addition to the routine monitoring for clinical signs as appropriate for the procedure. Where any animal shows abnormal behaviour this animal is placed on enhanced inspection. Study animals are often weighed at regular intervals to detect early clinical signs.

We have written standard operating protocols for all our models including humane clinical endpoints and refinement procedures (e.g. timepoint studies). All facility staff will be fully trained in these models and signed off when proficient in the clinical signs of the relevant cancer model.

We always refer to previous studies for adverse effects of anti-cancer therapies and when a group is given a treatment for the first time, we initiate the study with a small number of animals (n=3-6) which is closely monitored before extending to a larger number. Where possible we use the least invasive method of drug administration.
Animals who start to show any of the specific symptoms of the tumours they carry may be given analgesia if the application of such agents does not affect experimental outcomes, other animals within the cage, or the ability to rear young (although it is not our intention to routinely breed from tumour- bearing animals).

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

To ensure the work in this licence is conducted to the highest standards and can be reproduced by other scientists, the ARRIVE guidelines will be followed at all times.

The risk of infection or delayed healing is minimised by following the LASA guidelines on aseptic procedures (LASA 2017 - Guiding Principles for Preparing for and Undertaking Aseptic Surgery).

The animal facility has full AAALAC and ISO9001-2015 accreditation. To conform to these standards, we must work to a high level of quality control on all fronts including husbandry, phenotyping and administrative processes.

We adhere to the Workman Guidelines for the welfare and use of animals in cancer research and regularly review the NC3Rs website.

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

We continually review our processes and take advice from the Named Veterinary Surgeons, Named Training and Competency Officer, Home Office Inspectors and the NC3Rs website. Our technical staff are very proactive in adopting 3Rs advancements such as non-aversion handling and single-use needles and contribute/attend events such as 3Rs workshops and Laboratory Animals Science Association (LASA) conferences.

8. Building a smart nerve fiber

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

Brain, Nerve, Neuron, Glia, Zebrafish

Animal types	Life stages
Zebra fish (Danio rerio)	embryo, neonate, juvenile, adult

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim of this project is to understand the cellular and molecular mechanisms by which the nerves in our brains are formed during early development and can adapt to life experience, using zebrafish as a model organism. This work will help understand not just healthy brain formation but also how disruption to the normal development of nerves contributes to human neurological diseases.

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

Why is it important to undertake this work?

The formation of our brains during early development is an extremely complex process whose underlying mechanisms are poorly understood. Even after development, our nerves are "smart" and can adapt to our life experience. This enables us to respond to our

changing environment, but how nerves achieve this is also not entirely understood. It is important that we elucidate these underlying mechanisms because disruption to nerve development or adaptability can lead to human neurological conditions. These include neurodevelopmental disorders such as schizophrenia, autism-spectrum disease or certain types of epilepsy. In order to cure or ameliorate the devastating symptoms of these diseases, which are a major societal burden, we need to understand which parts of the developmental process of the brain are disrupted and on which parts we can intervene therapeutically. Our lack of understanding stems partly from the great diversity of cell types in our brains and their huge and complex interconnectedness. It is their concerted interactions and communication that drive the formation of the brain and also enable it to respond dynamically to the environment. In humans, the nervous system is composed in equal parts by nerve cells called neurons and support cells called glia. Glial cells, in particular the type that insulate our nerves, have emerged in recent years as a key player in nerve formation and adaptability, but how they do this is unclear. This is partly because it is challenging to visualize the interactions between neurons and glia in detail, in real time, in traditional animal models, during and after brain development. To understand the signalling and communication that occurs between nerves and glia during development, in health and in disease, we will use zebrafish as the simplest vertebrate animal model organism that contains insulating glia. They have unique experimental advantages that enable us to analyse intact cellular interactions in a non-invasive manner and, crucially, the formation and operation of the neurons and glia that make up their nerves are remarkably similar to those of mammals like us.

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

Our long-term goal is to elucidate how the human brain works and to help find treatments for neurological disorders of the human nervous system. This work will inform us how cells in our brain cooperate to build our nervous system and inform us about what goes wrong when certain aspects of cells and their communication are disrupted. We will learn how cells respond to such disruption, and we will create new models of human diseases. In general, a better understanding of the processes and mechanisms underlying formation and/or disruption of the nervous system may reveal key molecular targets that serve as entry-points for future therapeutic discovery studies. The majority of the findings deriving from this project will be peer-reviewed publications published in open-access scientific journals. In addition, the new disease models we generate as well as new genetically altered zebrafish such as transgenic reporters are also important tools for further neuroscience research endeavours, and we will share these with the broader scientific community. We will prepare press releases and social media outputs to disseminate our work to the public in an easily accessible manner. New information will also be conveyed through presentations at scientific conferences and other public engagement opportunities.

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

Several groups will benefit from the outputs of our studies: the neuroscience research community will benefit from the newly generated knowledge on brain development and disease that can be integrated into our growing understanding of the nervous system. Furthermore, through training and supervision, personnel engages in this project e.g. doctoral students and postdoctoral researchers will benefit by learning how to leverage the zebrafish model organism to conduct scientific research. This will foster the next generation of biomedical scientists in the United Kingdom. As our work could bring us closer to treatments to cure or ameliorate human disease, patients, families, carers, and

pharmaceutical development efforts will benefit from our research. Furthermore, throughout the project, through dissemination of our work, the wider community will benefit from knowing that major areas of unmet need in human health are being tackled.

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

We have established internal and external collaborations with other researchers that employ other animal models, such as mammalian organisms, or other technological approaches to study related neuroscience questions. Our collaborative efforts will enable us to test the conservation of the findings of this project and open up new biological processes and molecular mechanisms for investigation. We will make our findings available through pre-prints and open-access publications, and will regularly present ongoing work at local, national and international scientific conferences, as well as social-media based outputs. Together with preparing press-releases, this ensures the widest possible dissemination of our work.

Furthermore, we plan to engage with specialized research funding specialists to establish collaborations with pharmaceutical partners to help increase the impact of the most translational aspects of our work.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 35500

Predicted harms

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

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

We aim to study the formation and function of the healthy nervous system and to assess how dysregulation of certain aspects of nervous system formation contributes to neurological diseases. Neurodevelopmental diseases represent a major societal burden, currently with very few treatments available, the majority of which target disease symptomatology, rather than causes. This is due to the complexity of the nervous system and the technical challenge of observing key biological events in real time in most animal models. To overcome these challenges, we use zebrafish as a model, since these vertebrates share remarkable genetic and cellular conservation, in terms of their nervous system development, with humans. This means that often their underlying disease mechanisms and processes are also similar, and thus zebrafish can be used as a relevant proxy to study brain development and malformation. We principally employ young developing zebrafish in the laboratory, due to their small size, optical transparency, and fast development – e.g. with a simple but functional nervous system within days of fertilization. Together with their high fecundity, which greatly facilitates certain types of genetic and pharmacological experiments, and with our ability to create transiently genetically altered animals, these advantages mean that we are able to carry out highly informative experimental procedures on brain development and brain function before the stages at which the animals become protected (five days postfertilization). Crucially, we do this entirely non-invasively, as the transparency of young zebrafish enable us to perform live-imaging to observe cells interact and tissues develop in real time, at very high resolution, while the animal is simply anesthetized. Thus, zebrafish enable us to gain

insights into biological events and disease processes that would be difficult to achieve with other systems.

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

Most animals used in our project will be genetically altered zebrafish with fluorescent proteins in cells or tissues of interest that allow us to track biological events in real time using a range of cutting-edge microscopes. In addition, many animals will also have further genetic alterations that change the function of specific genes of interest, e.g. a gene associated with a human disease. The combination of genetic alterations that allow us to see cells of the nervous system in the context of manipulation of specific gene function allows us to gain great insight into how specific genes affect nervous system formation, function and dysregulation. In many cases, we will also treat zebrafish with chemical compounds to assess how they affect the animal, and in particular the formation and health of its nervous system.

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

Due to the small size and rapid external development of young zebrafish, we can perform most experiments in a non-invasive manner, and with little and often no signs of adverse effects on the animal. We are, however, interested in treating diseases of the human nervous system, which requires the creation of models of certain aspects of human diseases using zebrafish. These include for example the introduction of human mutations in certain zebrafish genes expected to disrupt nervous system formation or function. In some cases, such animals can exhibit adverse effects including impaired development or disrupted locomotion. In most cases, we can study animals with such adverse effects at stages prior to their being considered sentient enough to experience any suffering, and prior to becoming protected.

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

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

We do not study animals that exhibit severe adverse effects. Occasionally, we maintain animals that exhibit moderate adverse effects, due principally to genetic alterations, because very few of our experimental protocols elicit adverse effects of their own. Such moderate effects may be due to disrupted brain development and manifest e.g. as impaired locomotion, or as increased stress. We would only study animals experiencing moderate adverse effects for short periods of time. However, even brief analyses of zebrafish can be very informative, due to their rapid development and the ability to directly watch biological events in the animal in real time. We expect that less than 1% of all the animals that we will use during our project would experience even this level of effect.

We also study animals with mild effects. Such effects could be manifest as subtle deficits in behaviours, or mild stress due to being restrained during microscopy. We expect that up to 5% of the animals that we will use might experience this level.

Our experience to date indicates that the vast majority (>90%) of animals will not exhibit evidence of experiencing an adverse effect that is observable. The vast majority of animals

that we use are for breeding and maintenance reasons, and we do not keep animals that exhibit any significant evidence of suffering for these purposes.

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 incredible complexity of our nervous system cannot be replicated in non-animal alternatives such as cell cultures. Brain function emerges from the concerted interaction and communication between many different types of cells, including nerve and glia cells, organized in complex three-dimensional structures that are formed and refined and continuously communicate over a protracted period of maturation. These are impossible to replicate accurately without recourse to animal models, for example in a petri dish. The consequences of disruption to the nervous system, for example through genetic mutations which trigger very complex cellular responses and interactions, cannot easily be reconstituted without animal models. Therefore, we need to use animals for this project. However, in using zebrafish, we make use of arguably the simplest vertebrate model in which the complexity of the nervous system can be directly interrogated.

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

Certain aspects of neuron and glial cell early development can be studied without using animals, by studying cells grown in the dish, which has provided many important insights. However, this project aims to use the simple zebrafish model to study the complexity of brain formation, function and disruption as it occurs in the natural setting. At present there are no other suitable non-animal alternatives to do so. In the future, cell culture techniques may eventually allow miniature brain-like "organoids" to develop in a petri dish that sufficiently recapitulate the complexity of interactions between neurons and glia. In years to come such technologies may become so refined that we they can be used to study neuron and glial cell interactions and neural circuit function, and we look forward to incorporating such models into our work, but at the moment zebrafish provide the most powerful model to study intact dynamic interactions between cells during brain formation and function.

Why were they not suitable?

While brain organoids are beginning to show great promise as an experimental tool for neuroscience, they have not yet been established to the point where they have all of the neurons and glial cell types that would be required to be able to probe the mechanisms of nervous system formation, function and dysregulation.

Reduction

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

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

We have calculated the number of animals that we are likely to use based on our current use of zebrafish as a model system. One of the major advantages of using zebrafish as an animal model is that many distinct genetically altered lines of zebrafish can be maintained by single laboratories. The vast majority of protected animals will be used for the purposes of maintaining (protocol 1+2) and generating (protocol 3) genetically altered lines to visualize or manipulate neurons or glial cells. We estimate that we will need to generate/maintain up to about 100 different lines including transgenic lines (reporters and effectors) and mutant lines with targeted gene disruption (including in combination with transgenic lines). We typically maintain of three separate tanks per line, with an average of 20 protected animals in each, in order to ensure generation of sufficient offspring for experimental purposes as well as for keeping back-up stocks. As we aim to replenish stocks once per year, we will use 5 separate generations of each stock during this project, yielding a total of approximately 30,000 animals. We will maintain these genetically distinct lines of fish and where possible share them between several users to maximize their output, and constantly monitor our stocks to ensure that we are not maintaining lines that are not in use.

Importantly, we are now able to assess any effects of new reporters or new gene manipulations (e.g. originating from crispr-cas9 gene editing) early on in development, very soon after injecting the relevant reagents, during unprotected stages. Previously, if we were interested in a gene's function for example, we would have to edit the genome and grow animals up to sexual maturity, maintain them through subsequent generations, and test if they affected a biological process of interest. Now this can be achieved within days of such "editing" which allows minimizing the duration of the period that animals need to be maintained, representing an experimental refinement. The ability to target gene function directly means also that we can quickly assess how individual genes affect many different biological functions. For example, we can see how editing different genes affects different cell types or disease states because we can directly edit genes in animals with fluorescent reporters in their neurons, or glial cells, or other types of cells. These improvements allow us to only generate stable mutant lines (i.e. raise animals into protected stages/adulthood) from genes that exhibit particularly important functions when assessed by acute gene editing.

In addition to the 30,500 gene-edited animals from protocols 1-3, we predict using up to a further 5000 protected animals in our experimental analyses using protocol 4. The vast majority of analyses using the approaches in this protocol will consist of high-resolution live-imaging experiments and will use unprotected embryonic forms prior to 5 days postfertilization, before zebrafish are considered sentient enough to require legislative protection. Occasionally we may need to assess certain phenotypes and processes at slightly later stages between 5-15 days postfertilization, but these will be a minority of experiments. Throughout the work using this protocol we will carry out careful calculations to define how many animals are needed to find statistically meaningful effects in our experiments, as we have previously done.

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

The main step that we take in our experimental design is to determine what questions we can address using zebrafish at unprotected stages. The use of zebrafish at unprotected stages greatly reduces the number of animals on experimental protocols, and we will continue to pursue this strategy. However, the modelling of disease and the analysis of some later aspects of neural circuit maturation are not possible to carry out at unprotected stages, nor are breeding and maintenance protocols, nor the generation of new stocks of genetically altered lines. Nevertheless, we can assess the efficiency of transgenesis and gene editing at unprotected stages, which reduces the number of animals taken on to protocols. Another important way in which we can reduce animal number is through live imaging of individual animals over time. Through time-course or time-lapse imaging, we can gain a wealth of information about the dynamic nature of biological events from single animals that would otherwise require multiple animals being assessed at many different time-points. Furthermore, we can combine multiple reporters within one animal (e.g. with different fluorescent colours) to monitor multiple processes or cell types at once, reducing the number of animals needed - as the alternative would be to carry out analyses separately on several distinct reporter lines.

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

We will continue to work to optimise the efficiency of transgenesis and gene editing, particularly cell- type specific gene editing, which we hope will reduce the number of animals that we use in our work. We will also work closely with our aquarium staff who are implementing trials that aim to adapt husbandry procedures to ensure more reliable sex ratios in our breeding stocks. Skewed sex ratios are prevalent in zebrafish stocks and definitive protocols to balance male and female generation have not yet been established. This would help reduce the number of animals that we need to use to generate sufficient breeding stocks for our experiments. In many cases, we will also be able to carry out pilot experiments on zebrafish that can be shared by other users who have the authority to do so.

Refinement

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

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

We use zebrafish to study neuron-glial interactions during nervous system formation, function, and dysregulation. We use zebrafish due to the ease with which one can generate and maintain genetically altered animals with fluorescent reporters that allow direct visualisation of molecules and cells of the nervous system in embryonic and larval stages without the need of invasive procedures, and typically without any evidence of adverse effects. We also use zebrafish because of the ease of gene editing and

expression. Again, the ability to carry out transgenic manipulations and gene editing on newly fertilised eggs means that their efficiency and any potential adverse effects can be observed prior to their development to ages that are protected. This is an important refinement that reduces animal numbers used and helps reduce any potential suffering the animal might experience, were it not possible to do so.

Our principal experimental methods involve live imaging zebrafish. We can carry out high-resolution imaging of animals to assess how gene or compound function affect biological processes of interest, and in a manner that shows no signs of causing distress to the animal. We can also carry out extensive in-depth imaging over time of individual animals, either of the structure or function of their nervous system in a healthy or disease context. Time-lapse and time-course imaging of individual animals is a procedural refinement that enables us to reduce the number of animals needed per experiment to gather enormous amounts of information. Such analyses provide insight into dynamic processes impossible to gain in other systems that would require the use of multiple animals if using other models.

We continue to pursue any innovations in husbandry practices, and when trialled and deemed successful, will be applied to our protected stocks, and may significantly reduce numbers of animals used for breeding and maintenance throughout the project.

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

Myelinated axons are a vertebrate specific elaboration. Therefore, zebrafish are the simplest standard model in which they can be studied. Zebrafish also have an early onset of myelination and so are arguably less sentient than mammals during myelination, and we make every effort to study animals at the earliest stages at which we can address the questions that underpin the aim of our studies.

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

Very few of our experimental protocols cause significant harm to animals, and are already well refined. The main source of potential adverse effects to animals comes from the generation of new genetically altered animals where all possible effects on the animal are not possible to predict. However, as noted throughout, we can assess how new genetic alterations affect animals at unprotected stages and we carefully monitor animals following the introduction of new genetic alterations. We will also take advantage of a cutting edge stock management database that will allow us to better track and monitor survival rates and any effects seen across all of our stocks.

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

We follow the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines issued by the National Centre for the 3Rs, and will follow the recently published guidelines put together by a group of zebrafish researchers in collaboration with animal welfare experts at the Federation of European Laboratory Animal Science Associations (FELASA). In addition, we continue to refine practice across all experimental approaches as innovations and advances are published in the literature.

How will you stay informed about advances in the 3Rs, and implement these

advances effectively, during the project?

I follow zebrafish husbandry literature to keep abreast of the newest developments in best welfare practice, and also stay informed about relevant innovations through our proactive biological services unit at our establishment. I also follow NC3Rs on social media. We will implement appropriate advances through discussions with our local vets and named animal care and welfare officer.

9. Development of novel tumour-targeted nanomedicines for cancer therapy

Project duration

5 years 0 months

Project purpose

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

Key words

Cancer therapy, Gene delivery, Tumour targeting, Delivery systems, Nanomedicines

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 purpose of this work is to develop new delivery systems able to carry anti-cancer therapeutic nucleic acids (i.e. DNA, RNA, siRNA) and drugs specifically to the tumours, without secondary effects to normal tissues.

The aims of this study are:

to characterise novel drug and gene delivery systems

to determine the efficacy of the anti-cancer therapies delivered by these systems

to determine the ability of the delivery systems to reach the brain following intravenous administration (with the ultimate aim to reach and treat 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?

Effective cancer therapy remains an important medical challenge. At least 1 in 3 people in the UK will be diagnosed with cancer during their lifetime. Cancer kills more people in the UK than any other disease: one cancer sufferer dies every 15 minutes, which adds up to over 35 000 people a year. In particular, prostate cancer is the second biggest cause of cancer-related deaths in men in the United Kingdom, with a bleak outlook for men with advanced disease. Glioblastoma brain tumour is one of the most aggressive types of cancer, with only 6% of patients to survive for 5 years or more. Glioblastoma cells are often resistant to chemotherapy and radiotherapy, and can infiltrate the surrounding normal brain tissues, making the complete resection of the tumour impossible. Melanoma is fatal in about one- fifth of cases. The incidence of cutaneous melanoma is increasing worldwide at a rate second only to that of lung cancer in women, and it is the leading cause of cancer deaths in American women aged 25 to 29 years. Therefore, improved treatment of cancer would greatly reduce patient suffering and save many lives.

The efficacy of conventional therapy is often limited by the difficulty to selectively reach tumours after intravenous administration, without secondary effects to normal tissues.

Novel targeted delivery systems have the potential to be highly selective for the tumour and thus to significantly improve therapeutic responses after intravenous administration of the anti-cancer treatment.

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

This research is likely to lead to patentable results. The obtained results will significantly contribute to the translation of scientific findings into clinical benefit. If successful, the proposed approach should lead to clinical trials and could meet the industrial needs in the field of cancer therapy, by providing innovative, more efficacious treatments for cancer. This research should also result in publications in high-impact factor multidisciplinary journals.

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

Academia (short-term beneficiaries):

The proposed project is at the interface of cancer therapy, drug and gene delivery. As a result, it will be of interest to the thousands of academics investigating these topics worldwide. This will include the scientists involved in cancer biology, gene delivery, biomaterials, pharmaceutics, as well as clinical oncologists. The research will address the unmet need of tumour-targeted delivery of drugs and genes by intravenous administration, which is currently the subject of much research worldwide. Ultimately, if the project is successful, the possibility to use efficacious tumour-targeted nanomedicines would give a second therapeutic chance to many potentially life-saving therapeutics previously unable to reach the disease site, thus enlarging the therapeutic arsenal available for cancer therapy.

Academic benefits to the applicants and their collaborators arise from the developing collaborations that will result from this work, the culture change that will occur by working with researchers from complementary disciplines, the publications in high-impact factor multidisciplinary journals, the outreach engagements with the direct beneficiaries of the research and the general public. The research may also lead to patents, that will benefit

the applicants as well as the pharmaceutical industry.
To ensure that other academics can benefit from our studies, we will continue our commitment to publish peer-reviewed articles in high impact factor journals relevant to the field, and give presentations at national and international conferences.

Cancer patients (medium- to long-term beneficiaries):

Cancer kills more people in the UK than any other disease: one cancer sufferer dies every 15 minutes, which adds up to over 35 000 people a year. The proposed research will address the unmet need of tumour-targeted delivery of therapeutics by intravenous administration, which is currently the subject of much research worldwide. Numerous highly promising therapeutic drugs and genes cannot be used clinically because of their failure to specifically reach remote tumours by intravenous administration.

This represents a frustrating impasse, resulting in many potentially life-saving therapeutics being unavailable until this delivery issue is solved. The impact of our proposed project is therefore likely to be significant and would potentially have a major impact on healthcare, as there is currently no gene medicine commercially available for the intravenous treatment of cancer. Developing novel targeted treatments for cancer will not only kill the tumour cells but minimise the death of normal cells in the body. They will therefore reduce the painful side effects associated with conventional therapies and improve the likelihood of patient survival in the long term.

Pharmaceutical industry (medium- to long-term beneficiaries):

The pharmaceutical industry will be another beneficiary from this research, as this project aims to tackle a scientific challenge with direct relevance to patient treatment. The development of a safe and efficacious nanomedicine able to specifically target and treat tumours after intravenous administration would have a major impact on healthcare. World gene therapy market is rapidly expanding. However, it is still in the experimental stage with success yet to be achieved in developing completely curative therapeutic drugs. To date, only two anti-cancer gene medicines, Gendicine® and H101® are commercially available. These two p53 tumour-suppressor gene medicines are delivered in a viral vector for the intratumoral treatment of head and neck squamous cell carcinoma, in conjunction with chemotherapy and/or radiotherapy. There is currently no gene medicine commercially available for the intravenous treatment of cancer, thus making the development of an intravenously administered tumour-targeted gene medicine particularly promising. The proposed project is expected to improve the efficacy of intravenously administered gene therapy to tumours, leading to better anti-cancer therapy.

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

We will seek intellectual property protection where appropriate to ensure the commercial usefulness of the findings downstream and to maximise exploitation potential of this work. Manuscripts describing the results obtained will be submitted for publications in high-impact factor multidisciplinary journals as soon as possible during the project.

The applicant is fully committed to explaining their research aims and results with the general public. Every suitable opportunity will be taken to describe the research work and its rationale to the public. Our research activities (publications, presentations at conferences) will be promoted online on our university webpage and our personal research webpages.

Species and numbers of animals expected to be used

- Mice: 1050

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 will be used in this project.

To date, the novel therapies have been tested on cancer cells *in vitro* with encouraging results. However, a whole organism bearing a tumour is necessary to verify the delivery of these therapeutics to the tumour and the absence of any side effects.

Mice are the species of choice for these studies because they demonstrate many features of the human diseases and the genes involved are common to both species. Specifically, we use athymic nude mice because they are immunocompromised and hence tolerate growth of “foreign”, human (tumour) cells (xenograft). Nude mice are commercially available and are routinely used for these types of studies.

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

New delivery systems will be extensively tested in cell culture and only those of proven efficacy are advanced to *in vivo* studies, first to establish suitable dosing, then to evaluate their biodistribution. The delivery systems showing suitable biodistribution will then be tested for efficacy in a tumour-bearing animal. Efficacy will be measured as tumour growth delay, by calliper measurement and/or imaging of subcutaneous tumours. A pilot study with just a few animals will indicate if further work would be appropriate.

Animals will be housed in groups in cages with soft bedding and environmental enrichment (i.e. plastic houses). Good husbandry, daily monitoring and care by a team of well-trained animal technicians will ensure that animal welfare is paramount.

In all experiments, the mice will be monitored closely to ensure that no unforeseen adverse reactions cause distress to the animals. Tumours will be established in mice by a single subcutaneous injection of cancer cells and allowed to grow until they reach a suitable size for distribution and therapy studies. Tumour growth will be measured before and after treatment to determine the response to the novel therapy. The mice will be injected intravenously with the treatment up to once a day for up to 15 days. The mice will be closely assessed for any (rare) signs of health problems. We will take every measure to avoid any animal suffering. Following humane killing of the animals, tissues such as liver, lung and tumours will be removed for analysis.

Animals will normally be on study for only 2 months.

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

Drugs will be given at levels expected to be non-toxic from other studies / literature. Increasing doses of novel anti-cancer agents will be given starting from the lowest known therapeutic dose as determined in other studies. The risk of adverse effects is minimised by choosing small incremental steps for the dose escalation and close monitoring of the animals. We have many years of experience working with this model and have hardly ever seen any adverse effects.

Nevertheless, animals may potentially show signs of acute toxicity (e.g., extreme pallor, circulatory collapse, respiratory distress, depression/excitation…) which will trigger immediate euthanasia in the very rare event of it appearing. Signs of chronic toxicity (e.g., weight loss, piloerection, hunched posture) and/or localised tissue damage (e.g., phlebitis, skin irritation or ulceration) are also possible, although very rare. If present, these adverse effects are expected to be short-lived (some just hours, a few a maximum of a few days) and will be minimised and controlled as advised by the NVS and following clear end-points in the licence .

Whole body imaging of the mice is not an invasive procedure, but the animals need to be still during the scanning session and therefore an anaesthetic is required. The level of anaesthesia will be maintained at sufficient depth to achieve light general anaesthesia. During scanning, animals will be kept warm by means of a heated platform. They will be monitored via the screen; anaesthesia is monitored by observing body movement.

Repeated general anaesthesia is stressful to the animal, therefore, the number and length of imaging sessions will be kept to the minimum possible to answer the specific experimental questions. Similarly, the longest possible interval between anaesthetics (to allow best recovery) will be used, depending on the experiment.

If a study requires more than one imaging sessions on the same day, mice will be given a treat (i.e. Nutella) after the imaging session as a means of positive reinforcement to minimize the stress from the anaesthetic 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)?

The maximum severity for both protocols in this licence is Moderate but we expect around 70% of mice to possibly experience this severity and around 30% to experience only a Mild severity.

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

- Killed

Replacement

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

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

To date, these novel therapies have been tested on cancer cells *in vitro* with encouraging results. However, a whole organism bearing a tumour is necessary to verify the delivery of these therapeutics to the tumour and the absence of any side effects. For biodistribution studies and drug delivery, the murine model is the model of choice, as the mouse physiology, in terms of organs and systems, has many parallels to that of human systems. Moreover, athymic nude mice do not reject human cancer cells (xenografts) and thus provide the ideal living system to test new drugs targeting these cells.

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

We have fully considered alternative approaches such as computer modelling and use of non- protected species such as nematodes; we have also searched non-animal alternative databases.

Why were they not suitable?

At present, it is impossible to recreate the biological environment in terms of biodistribution throughout complex physiological systems following the administration of a therapeutic agent. A whole mammalian organism is necessary to verify the delivery of these new therapeutics to their target, the absence of any unspecific distribution, the general toxicity which could eventually occur, as well as any changes in the behaviour of the animal as a result of the treatment.

Reduction

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

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

New delivery systems will be extensively tested in cell culture and only those of proven efficacy are advanced to *in vivo* studies, first to establish suitable dosing, then to evaluate their biodistribution. The delivery systems showing suitable biodistribution will then be tested for efficacy in a tumour-bearing animal. From previous studies, we determined that having 5 animals per treatment group would lead to statistically significant results. A study comprising the novel nanomedicine to investigate and its controls, done on 2 cancer models, would therefore require 50 mice. We would expect to do 2 full investigations per year, for 5 years, leading to a maximum of 1000 mice for the evaluation of therapeutic efficacy. 50 mice will be used for the determination of dose ranging. These numbers were estimated from previous experiments in my previous Project Licences.

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

The *in vitro* experiments were drastically optimized in such a way that we moved to *in vivo* work only when we had a very high chance of getting positive results in mice.

We used the NC3R's Experimental Design Assistant to reduce the number of animals being used in the project.

Pilot studies using just a few animals (1 or 2 per treatment) will indicate if further work would be appropriate.

From our previous studies, we determined that having 5 animals per treatment group would lead to statistically significant results. In the past, we have had guidance from the University's designated biostatistician, and we have consulted with the current one for this project as well.

The conditions of tumour formation and some dose ranging for some drugs entrapped in the novel drug/gene delivery systems have already been determined by using my past Project Licence and will not require the use of new animals.

IVIS/Quantum GX2 microCT imaging will be used for analysing tumour regression in mice bearing luciferase-expressing tumours and for analysing gene expression in mice injected with DNA-encoding luciferase. These experiments will further reduce the number of animals needed for one given experiment, as the level of gene expression could be measured at various times without having to sacrifice some animals for each time point.

Multiple outcome measures will be assessed in the same animals (i.e. imaging of tumours using the IVIS/Quantum GX2 microCT, measurement of the tumours by calliper measurement, quantification of gene expression in the tumours and organs), resulting in a decrease of the number of animals needed whilst obtaining statistically different results suitable for publications.

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

The number of mice to use in my project will be reduced as much as possible by optimising the delivery systems *in vitro* as much as possible, only progressing to *in vivo* studies if they have a very high chance of success.

Pilot studies will indicate if further work would be appropriate.

Previous knowledge of a suitable number of mice to be used to obtain statistically significant results will be used to minimise the number of animals used in each study.

The use of IVIS/Quantum GX2 microCT imaging for analysing biodistribution, tumour regression and gene expression in mice will be used as much as possible to further reduce the number of animals used in the project.

Multiple outcome measures will be assessed in the same animals, to decrease the number of animals needed in the study.

Refinement

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

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

Each experiment involving animals will be carried out in a way to minimise as much as possible animal suffering.

The mouse has been chosen for these experiments, as it is a well-characterised model for biodistribution and gene expression studies. Mice are the species of choice for these studies because they demonstrate many features of the human diseases and the genes involved are common to both species. We have extensive experience of this animal model acquired during our previous experiments.

Athymic mice will be used extensively as they have a proven track-record in the field, tolerating exogenous tumour implants to grow in a controlled manner, without hardly any associated adverse effects. They will be kept in suitable barrier housing to protect them from unwanted infections.

Methods which cause the least harm to the animals and which are the most likely to produce satisfactory scientific results have been chosen. The use of imaging techniques such as bioluminescence to monitor tumour development and to evaluate the targeting of new therapeutic systems to tumours is a significant refinement of experimental technique. We are continuously seeking new techniques that could cause the least distress to the animals.

The *in vitro* study of the cytotoxicity efficacy of any new therapeutic system on cancer cell lines, prior to any *in vivo* experiment, allows us to obtain essential data for choosing earlier endpoints, reducing the administered doses and the injection frequency, in order to cause the least suffering, distress and lasting harm to the animal.

In all experiments, the mice will be monitored closely to ensure that no unforeseen adverse reactions cause suffering to the animals. Tumours will be established in mice by a single injection of cancer cells and allowed to grow until they reach a suitable size (typically around 0.5 cm) for distribution and therapy studies. Tumour growth will be measured before and after treatment to determine the response to the novel therapy. We follow the UKCCR Cancer Research Guidelines and hence tumours never exceed 1.2 cm. The mice will be closely assessed for any (rare) signs of problems. We will take every measure to avoid any animal suffering.

At the end of the experiment, the most humane and appropriate method of euthanasia will be chosen.

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

The mouse has been chosen for these experiments, as it is a well-characterised model for biodistribution and gene expression studies. Mice are the species of choice for these studies because they demonstrate many features of the human diseases and the genes involved are common to both species. Using terminally anaesthetised animals would lead to an increase in the number of animals to be used, which is not in line with the 3Rs.

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

Provision of an enriched environment in animals' cage (i.e. plastic "igloo" or cardboard roll in which to hide, presence of nesting material, deep layer of mouse bedding) has been demonstrated to reduce the level of stress of the animals and to improve their quality of life, and this is our common practice. Good husbandry, daily monitoring and care by a team of well-trained animal technicians, with NVS support, will ensure that animal welfare is paramount. The procedures I will be using will be refined to minimise any harms to the animals by increasing the monitoring of the animals when performing a new technique. For example, cancer cells are normally injected subcutaneously and not surgically implanted; this route and method is much less painful and invasive than surgical implantation or some other injection route. Dosing with novel therapeutic agents will also aim to use the least invasive route (e.g., oral) but we will also need to use other routes (e.g., intravenous), given the need to mimic dosing regimens used in human patients and the fact that we need to investigate circulatory biodistribution.

The total number of injections will be the least possible to demonstrate an effect (usually no more than 10). When animals undergo imaging, the number of times and the time spent under anaesthesia will be reduced as much as possible and animals will be kept hydrated and warm. The post-procedural care will be refined by providing the animals with sugary treats (i.e. Nutella) following anaesthesia or any injections. Male mice housed in the same cage will be closely checked to prevent any fights. In the occurrence of significant fighting, animals will be separated. Only highly trained staff will perform experimental interventions.

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

I will follow Workman *et al.* Guidelines for the welfare and use of animals in cancer research (https://www.nature.com/articles/6605642). I will also follow best practice guidance from the Home Office, the National centre for the Replacement, Refinement and Reduction of Animals in research, the ARRIVE Guidelines, our own University guidelines, as well as publications reporting improved practice in animal studies in specialised, high-impact journals from my research area.

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

I will stay informed about advances in the 3Rs, by accessing information from the resources library in the website of the National centre for the Replacement, Refinement and Reduction of Animals in research. New guidelines and information about the 3Rs are regularly circulated by email by our NIO and by the Home Office. Regular training for PPL and PIL holders about *in vivo* work is organised within the Department by NACWO, NVS and NTCO. Staying informed about the most recent updates about the 3Rs regulations, good practice techniques and regulations, and attending the training sessions in the Department will allow me to implement these advances effectively during the project.

10. Investigating the effects of chronic kidney disease on cardiac structure and function

Project duration

5 years 0 months

Project purpose

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

Key words

Chronic kidney disease, Heart disease, Arrhythmia

Animal types	Life stages
Mice	adult

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

This project licence will characterise the pathways by which chronic kidney disease leads to cardiac dysfunction and identify novel therapeutic interventions to prevent or reverse these changes in the heart.

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

Why is it important to undertake this work?

Chronic kidney disease affects over 10% of the UK population and is a major but under recognised risk factor for cardiovascular disease. As chronic kidney disease progresses, there is increasing cardiovascular mortality due mainly to heart muscle disease – called "uraemic cardiomyopathy" – rather than atherosclerotic coronary artery disease. Previous work by this group and others has established that this heart muscle disease begins at an early stage and worsens in parallel with declining kidney function. There are currently only

a few recognised treatments that delay but none that prevent the onset of uraemic cardiomyopathy in chronic kidney disease. Mechanisms of disease and treatment are unclear.

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

We will publish the data in relevant academic journals and present at national and international conferences. The studies may shed light on mechanisms driving cardiovascular dysfunction in patients with chronic kidney disease and demonstrate utility of the therapeutic interventions for treatment of kidney dysfunction on the resultant cardiac structural, mechanical and electrical dysfunction.

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

Short to medium term we expect that the knowledge gained from this project will be shared with the scientific and clinical community and published in peer reviewed journals (output expected during the project). Thus the cardiovascular research community will be the primary benefactor of this work. The long term benefit of the work will be a better understanding of the mechanisms by which chronic kidney disease affects the heart and whether these pathogenic mechanisms can be prevented using the standard or novel therapies. This will open new avenues to reduce cardiovascular disease risk for this patient population for example reducing atrial or ventricular arrhythmias (output expected some 1-5 years after the project), with the ultimate aim to prevent or ameliorate high cardiovascular morbidity and mortality.

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

Publication in the relevant scientific literature and presentation of the data at international meetings is likely to be the primary output for this work. This includes negative data. The intention is that this work generates valuable datasets that will inform clinical practice and may provide a novel therapeutic avenue for the treatment of cardiac dysfunction in patients suffering with chronic kidney disease.

Species and numbers of animals expected to be used

- Mice: 700

Predicted harms

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

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

Mice are the lowest mammalian species with sufficiently similar kidney and cardiovascular physiology to that of humans, allowing transfer of findings to man. Human cellular models do not replicate the complex interaction between the kidney and the heart. Adult mice will be chosen to minimise variability and to allow full development of kidney structure and function.

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

Adult mice will receive a modified diet (adenine supplementation) which will lead to slowly progressive reduction of kidney function over several weeks.
Some animals (less than 5%) may undergo minor surgery to implant an osmotic mini pump or equivalent device under the skin to chronically deliver drugs or vehicle control. We will however always seek to deliver drugs orally (via drinking water, in food or by oral gavage) or by injections (intraperitoneal, subcutanous).

Some animals may undergo non-invasive assessment of structural and functional changes to the heart. This will include echocardiographic imaging and ECG monitoring under anaesthesia before recovery and ECG and blood pressure monitoring in conscious animals.

Blood sampling may also be carried out to measure any circulating factors influenced by the kidney disease or drug administration. In the majority (if not all of the animals), the heart will be excised under terminal anaesthesia for further Langendorff perfusion or cell isolation for assessment of cardiac and cellular function to maximize the data obtained from each animal.

Anthropometric measurements will be taken (weight, tibia length).

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

Reduced kidney function does not cause pain for animals, but may cause lower food intake and associated weight loss. These effects are expected to become more pronounced the longer the animal is maintained on the altered adenine diet. The maximum duration for an animal to receive the altered diet is 20 weeks. Body weights of animals on modified diet will be monitored regularly and animals will be observed for altered behaviour. If an animal loses weight rapidly, stops eating or displays persistent abnormal behaviour, it will be removed from the experiment and humanely killed. Pilot experiments will determine whether adenine content (%) can be reduced or whether duration on adenine diet can be reduced, allowing development of kidney and cardiac dysfunction whilst reducing adverse effects.

Based on published data, the doses of the proposed therapeutic agents to be delivered under this project license have not been shown to cause any significant side effects in the mice or patients.

The therapeutic agents administered (e.g. SGLT2 inhibitors, MRAs, sodium channel blockers, sodium pump anatgonists, sacubitril/valsartan and others) will not exceed the LASA recommended maximum volumes for dosing. The safety profiles when injected subcutaneously or intraperitonealy in animals is well documented. However, if animals display unexpected adverse effects, then they will be humanely killed.

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

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

20% of animals will be non-recovery

80% animals will experience moderate severity.

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

- Killed

Replacement

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

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

Kidney function and its effects on cardiac mechanical and electrical function is complex, involving the interaction of multiple factors, including many cell types and physical and neurohormonal local controls, that cannot currently be studied without animal models. Human cellular models cannot fully recapitulate the complexities of the kidney/vascular/cardiac interactions. Our understanding of the processes involved, and their relative importance, limits our ability to use computer modelling, though this is a goal we are working towards.

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

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

More recently, in our lab we have started reprogramming human induced pluripotent stem cells (hiPSCs) into cardiomyocytes and fibroblasts and these will be used to investigate the effects of adipocytes in vitro, in parallel with the experiments proposed in this project License. It should be noted however that traditional 2D (or indeed 3D) culture models fail to fully recapitulate complex functional biology, hence the need for the use of mouse models. Furthermore, we also use patient electroanatomical mapping data

Why were they not suitable?

Stem cell-derived cardiomyocytes, either in 2D or 3D culture, do not fully recapitulate the complexity of the heart, including many cell types and physical and neurohormonal local controls. Furthermore, mimicking complex interaction between the kidneys and the heart is currently not possible in vitro.

Human patient data acquired during electroanatomical mapping procedures is usually acquired from patients that already have cardiac arrhythmias so whilst these are very valuable tools for studies, they do not allow for investigation of earlier stages of disease development.

Reduction

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

numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

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

Expected effect size will be determined through consultation of the literature, previous experience of techniques proposed or through small pilot experiments when possible.

We have used statistical methods to calculate how many animals we need to get meaningful data where possible. This was determined based on the pilot data generated by us and our collaborators using the adenine diet models, and a number of different therapeutics and dosage concentrations we plan to investigate in vitro, on differential effects on atrial and ventricular chamber of the hearts.

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

When designing the experiments, we performed statistical analysis to ensure we use the minimum number of mice per group that will be informative. Importantly, we also employ a variety of approaches to reduce animal usage, including:

Performing and analysing experiments in a blind fashion where possible to reduce investigator- induced bias

Using standardised experimental methods to reduce variability

Optimising tissue usage through the use of innovative technology, such as RNAseq, optical mapping, novel cell isolation technique, etc..

Performing power calculations before any experiments to determine the required animal number to detect a change.

The NC3Rs EDA tool will be used to design experiments.

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

To maximise the information gained from a single animal we aim to perform multiple in vivo and in vitro analyses. Where possible, cell line work and in vitro manipulations have been designed to yield the maximum possible information and reduce animal use.

Better reporting of research should result in better science and more effective use of animals in experiments. Therefore, our findings will be reported (using the ARRIVE guidelines) in the scientific literature and at conferences, thereby minimising risk for future unnecessary animal experiments conducted by others.

Furthermore, literature will be continually reviewed to ensure that we are not repeating published work and that our hypotheses are based on the most up to date knowledge.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative

care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

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

Mice will receive dietary supplementation with adenine to induce gradual persistent reduction in kidney function and associated changes in the heart. These changes imitate the disease process of chronic kidney disease in humans. The effects of adenine on the kidney are controlled by the dose and duration of adenine exposure. This allows for fine-tuning the level of kidney impairment to the minimum required for the planned experiments, thereby avoiding undue harm. Furthermore, it means that changes for the animal are mild early on, building only gradually over time. Hence, the animal experiences more significant changes only for a limited duration in the late phase of the experiment. Effects of adenine are very specific on the kidney, as it is concentrated there. Direct harm to other organs such as muscle or liver is avoided. Existing surgical chronic kidney disease models are severe and involve surgical removal of 5/6th of kidneys during two surgical procedures, within a week of each other. Clearly patients do not develop chronic kidney disease in such a manner, thus the relevance of surgical chronic kidney disease models is questionable. Furthermore, adenine diet model spares animals from distress of anaesthesia and traumatic recovery periods that are common to surgical models of chronic kidney disease. A diet-based model is therefore scientifically superior to surgical models and more clinically relevant.

The use of osmotic mini pumps to deliver the drug of interest over a long period of time (up to 6 weeks) reduces the need for repeated handling and injections and therefore reduces the distress caused to the animal. Analgesics will also be given pre- and post-operatively as required.

Echocardiography will be carried out under general anaesthesia (up to 30 minutes) to prevent distress from physical restraintand and equally to produce better quality data. The apparatus for in vivo ECG/Blood pressure measurements is already refined to ensure the least distress to the animal i.e. tunnel used to secure animal in natural position and kept in dark throughout procedure. This experiment is done without anaesthesia as anaesthetic induction would be more stressful than procedure itself.

Blood sampling will follow published guidance on suitable volumes which can be taken while minimising harms to animals.

We are not planning on using novel (experimental) drugs. If a drug has not previously been used in our facility, we will consult literature for optimal delivery route, dose and duration, and then run a small pilot study, closely monitoring for any adverse effects. Specifically, we will consult literature on optimal dose and duration and this will be discussed with the Named Animal Care and Welfare Officer and Named Veterinary Surgeon to ensure animal welfare is maintained throughout the experiment and that minimum suffering is caused to acquire the scientific endpoints. We will also review each experiment on completion to see what lessons can be learned from the study in terms of endpoints (scientific and humane) and any animal welfare issues that may have arisen during the experiment that could then guide the next experiment.

Majority of follow up experiments are performed under terminal (non-recovery) anaesthesia. We will continue to make efforts to refine protocols and further reduce the welfare costs. Current best practices (e.g. needle sizes used will be kept to a minimum, use of minipumps to deliver therapeutics) will always be followed.

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

The mouse is the least sentient mammal that is most similar to humans in terms of the way their kidney and heart works. The research project examines dynamic interactions between organ systems, namely kidney and the heart, in the setting of persistently reduced kidney function. Lesser developed species (e.g. invertebrates) or aquatic life forms do not possess kidneys that resemble human kidneys and are therefore not a suitable model. To accurately model the changes in the heart associated with long-term kidney impairment, animals need to be studied over an extended period of time that precludes experiments on immature life stages or animals under terminal anaesthesia.

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

We reviewed the scientific literature extensively for mouse models of kidney disease and developed collaborations with other experts in this field. In addition to employing an evidence-based protocol, we will conduct a pilot study prior to proceeding to the full-scale experiment. This will introduce a distinct step at which further refinement can take place. Our protocol includes provisions to track changes with kidney function (serum creatinine and urea via blood sampling) and changes in cardiovascular function (echocardiography and electrical activity) over time.

For all procedures, the mice will be carefully monitored and if adverse events are observed, monitoring will increase in frequency and steps will be taken to alleviate them or the affected mice will be humanely killed.

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

We will use the PREPARE guidelines to ensure our experiments are planned and conducted in the most refined way. LASA guidelines will be consulted to ensure correct dose volumes are selected for the specific route of injection/blood sampling. We will also stay up to date with the NC3R website resources and guidance on the best and most refined practices. All the data will be published according to the ARRIVE 2.0 guidelines.

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

Regularly monitor the guidance given by the NC3R's website, making use of the online resources to ensure the project is carried out efficiently and using the best methods for animal welfare.

11. Neuronal cell development and survival

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Motor neuron disease, Amyotrophic lateral sclerosis, MND, ALS, Spinla muscular atrophy

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim of this project is elucidate the role of defective functions of the components of axonal transport in the onset of motor neuron 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?

Diseases of motor neurons affect adults and children. Amyotrophic lateral sclerosis (ALS; also known as motor neuron disease, MND) is the most common from adult onset motor neuron disease. ALS is a fatal disease affecting up to 5,000 adults in the Uk at any one time and it typically kills within 2-5 years from the onset of symptoms. Spinal muscular atrophy (SMA) is the most common childhood neurological disease affecting 1 in 6,000 – 10,000 children. It is caused by the loss of spinal motor neurons leading to wasting of muscles and loss of muscle movement. There are several types of SMA distinguished by their genetic and clinical features. A genetically dominant form of SMA, known as SMA-LED, has a preference for the lower limbs. Children with SMA-LED often present at birth with muscle tightening of the lower limbs and dislocation of the hips. Walking is delayed

and infants may require surgical intervention to correct the muscle tightening and hip dislocation. Some children with SMA-LED also present with malformation of the brain cortex which causes intellectual disability.

Working from the mouse phenotype to mouse primary cells and neurons is important for the understanding of the mechanisms of ALS and SMA-LED onset and progression. Using this knowledge in human induced pluripotent stem cell (iPSC)-derived neurons and fibroblasts and applying this information back to human conditions for cross species comparisons at the cellular and neuronal tissue levels will set a paradigm for the effective use of both the mouse and human-derived cells as valuable model systems for understanding the underlying molecular mechanisms of motor neuron degeneration.

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

Our study will generate cell and molecular biology data at the cellular and organismal levels, which will contribute towards our understanding of the mechanisms of dysfunction and death of neurons in general and motor neurons in particular in the diseases of motor neurons. A number of intracellular organelles and pathways have been identified to be targeted by motor neuron diseases. Our project will generate important data on the functions of these organelles/pathways in relation to the disease and the underlying molecular and cellular mechanisms that lead to the death of the neurons, utilising cellular and mouse models for neurodegenerative disease. We publish our data from this project in primary research paper and conference proceedings. In addition, we present our results in national and international conferences and in our outreach events including the events that I organanise for the East Sussex branch of the Motor Neurone Disease Association and Care Centre. In my role as the Advisor on Motor Neurone Disease Science I will discuss our research plans and outcomes with patients and carers in the local branch meetings and encourage debate about future directions.

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

The benefit from the outcomes of this study could be immediate, as our findings could inform the beneficiaries about the causes and basic mechanisms of the disease. In the longer term, understanding the roles of defective pathways studied in this research in development of the disease, will potentially have a significant contribution towards discovering novel drugs and more effective treatment of motor neuron diseases and perhaps other related disorders. Moreover, this study could provide improved knowledge of prognosis for informing patients and ensuring best possible care planning. Thus, our findings will benefit the scientific community and clinician with a broad range of interests in neurological conditions. In addition, this research will benefit patients and their families, who have been affected by motor neurone disease, hereditary motor neuropathies, and some cases of intellectual disability; and health professionals, who work with patients and their carers.

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

We will disseminate our results by publications in peer reviewed scientific journals and oral and poster presentations in national and international conferences. We will also use my laboratories Twitter account to highlight these results and communicate them to research scientists, clinicians, students, charity orgnisations such as the Motor Neurone Disease Association, My Name'5 Doddie Foundation, and the Dunhill Medical Trust.

Species and numbers of animals expected to be used

- Mice: 3100 Loa mice
- 40 wildtype 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.

Motor neuron diseases target motor neurons in the central nervous system and thus access to the targeted tissue is only limited to the post-mortem stage in humans, when we can obtain data about the very late stages of the disease. We therefore need mouse models, such as those used in this study, enabling us to have access to tissues at all stages of life. Moreover, sequencing of the mouse genome has revealed that ~99% of mouse genes have a homologue in the human genome and that for ~80% of mouse genes an analogous (orthologues) gene exists in the human genome. In addition, human and mouse have common biochemical pathways.

Because of the above properties several large international mutagenesis programmes as well as individual groups have produced mutant and knockout mouse strains that could serve as model systems for neurodegenerative disorders such as motor neuron diseases.

The mouse clearly does not have the same physiology as humans, but does, largely, share the same biochemical pathways as well as genes. Thus we can work with mutant mouse models of human motor neuron degeneration to highlight the proteins and pathology that are associated with the disease and find targets for treatment.

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

The animals in this project will typically be used for collection of tissues after udnergoing schedule 1 killing or for fixation of tissues by perfusion under terminal anaesthesia.

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

The mouse strain used in this study is known as legs at odd angles (Loa) which was generated in an ENU mutagenesis programme at the Mary Lyon Centre MRC Harwell. Heterozygous Loa mice show a reptilian gait when walking, signs of motor function deficit and neuronal pathology that recapitulate SMA-LED in humans, otherwise they feed and drink normally and have a normal life span.Homozygous Loa mice are not viable perinatally.

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

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

The expected severity of breeding Loa animals is mild to moderate as this will produce viable heterozygous mice with reptilian gait when they walk but normal life span. For collecting tissues from all genotypes, pregnant mice will be killed at mid-gestation by schedule 1 killing for collection of embryos, as this is the only stage that viable motor neurons could be collected.

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?

Motor neuron diseases target motor neurons in the central nervous system and thus access to the targeted tissue is only limited to the post-mortem stage, when we can obtain data about the very late stages of the disease. We therefore need mouse models, such as those used in this study, enabling us to have access to tissues at all stages of life including at the embryonic stage which allows us to isolate and culture viable neurons and embryonic fibroblasts.

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

We have generated induced pluripotent stem cell (iPSC) lines from Loa mouse strain, which we can differentiate into motor neurons for some of our cell biological assays. Although we have established the stemness of these iPSC lines, we will still have to characterise their functionality and *in-vitro* development and make isogenic wild type cell lines of these cells. In addition, we have patient-derived fibroblasts and iPCs. We will introduce key mutations identified in humans in these iPSCs, using CRISPR-Cas9 genome editing technology, for cross-species analyses of our findings and for some of the biochemical and cell biological assays.

Why were they not suitable?

We will still need to isolate primary neurons from mice to verify and expand our findings from human cells and/or iPSC lines, as these cell lines have gone through reprogramming techniques for conversion from fibroblasts into stem cells, which would then be differentiated into neurons. All these manipulations could have adverse confounding effects in the biology of the cells under study and thus it is important to verify our results from these cells in primary neurons isolated 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?

Mice will be used in this study to obtain embryonic fibroblast and neuronal cell cultures. As these primary cultures do not survive for long periods of time, fresh cultures and therefore mice will have to be used for this project. The estimate for the Loa mice is based on our previous use of these animals for providing tissues for our studies. For isolating motor neurons we will need to set up timed-matings for isolation of embryos at 13.5 dpc, which needs expansion of the colony from time to time in order to obtain successful matings for obtaining the tissue in a timely manner. The Loa mice are maintained as heterozygous animals by crossing with C57BL/6 wildtype strain. Thus, we will need 40 breeding C57BL/6 mice to use over 5 years (4 every 6 months).

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

As the mice in this project are used for collecting tissues, the numbers are estimates for maintianing a small colony of the Loa strain based on our previous experience.

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

We will seek to have as efficient a breeding programme as possible for obtaining the tissues that we need for the project from as few as possible mice. This will be achieved by keeping 3-5 breeding pairs at any one time, 8 heterozygous mice (male and female) for replacing breeding mice, and up to around 10 mice for timed-matings. As mentioned above, we might have to revise these numbers from time to time to ensure successful matings and timely access to the 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 be using the Legs at odd angles (Loa) mouse model in this project. The mouse strain was generated in an ENU mutagenesis project in Mary Lyon Centre MRC Harwell. The Loa strain has a point mutation in the heavy chain of cytoplasmic dynein and recapitulates spinal muscular atrophy lower extremity predominance (SMA-LED) in humans. The site of the Loa mutation is one and two amino acids from two mutations in humans which cause SMA-LED, making the Loa strain an excellent mouse model for investigating SMA-LED. This mouse strain is also defective in axonal transport, which is impaired in amyotrophic lateral sclerosis (ALS). The Loa mouse therefore provides an excellent model for investigating the role of defective axonal transport in the onset and development of ALS.

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

The complexity of motor neuron diseases necessitate the use of mice. Moreover, the high similarities of the mouse genetic and biochemical pathways to those in humans as well as the similarity of the presentation of the disease phenotypes to the human disease make this mouse strain a suitable model organism in this study. We will be using mouse embryos in most parts for isolating and culturing primary cells. Adult mice will be used for breeding, timed-matings, tissue collection.

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

As the Loa mice show mild to moderate phenotype and have a normal life span we don't expect major adverse effects from the Loa mutation. We follow best practice in animal husbandry with regular monitoring for detecting any animals that might be in distress.

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

We will follow the ARRIVE guidelines and any employ appropriate new developments such as the new initiative for developing a compliance checker to automatically assess scientific manuscripts to ensure transparency in our animal research in line with ARRIVE Essential 10.

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

I keep abreast of new developments in 3Rs by going through newsletters and emails which I regularly receive from NC3Rs. In addition, we are keen to utilise CRISPR-Cas9 technology and human derived iPSC lines as best as possible towards the replacement and reduction objectives of 3Rs.

12. Novel therapeutics for metabolic disorders

Project duration

5 years 0 months

Project purpose

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

Key words

Obesity, Diabetes, Drugs

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 aim of this study is to investigate the complex interactions responsible for the regulation of food intake and energy balance that occur between the gastrointestinal tract and the brain with the view to develop novel therapeutic for the treatment of metabolic disorders in humans.

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

Why is it important to undertake this work?

This work could lead to new anti-obesity drugs, and treatments for diabetes and other metabolic disorders such as anorexia nervosa and eating disturbances associated with

cancer. There is a huge clinical need for this research because of the global epidemic of obesity and diabetes.

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

Information and publications: A greater understanding of appetite regulation and modulators of metabolic rate. This research will be published in peer reviewed journals and presented at international conferences.

Use of information: As a research group, specialising in taking basic research discoveries from the scientific lab to the clinic to test their therapeutic use, we have taken five compounds identified from previous animal licences into Phase 1 clinical trials. Two of these potential drugs have been sold to pharmaceutical companies, and two have been licensed to a biotech for continued development. It is anticipated that building on our knowledge, our future research would identify therapies which would be advantageous for the treatment of metabolic disorders. A likely outcome in 5 years would be completion of a Phase 1 clinical trial for at least one potential drug identified and characterised on this project licence. As we continue to publish our research, we also expect others working in this disease space to learn from our research to aid their development of drugs.

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

Short term: Other researchers and organisations involved in metabolic research and development of drugs to treat metabolic disorders will have access to our research findings.

Medium term: Research will enable the organisation to identify drugs and their combinations to trial more effective treatment of metabolic disorders such as obesity and diabetes.

Long term: Clinical findings based on the scientific data produced by animal studies will impact on the development and licensing of new drugs.

Drugs that will be developed through this research will benefit patients and society. Treatment of obesity and the diseases which occur as a result of it, such as diabetes and cancer, costs the NHS £6 billion a year and is set to overwhelm clinical capacity of the health service. By reducing the levels of obesity, patients would lead a longer, healthier life with extended productivity preventing an unbalanced society.

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

We will share outputs of our research with the public via social media and through public events.

We will communicate our findings to other scientists through publications at scientific journals and presentations at meetings related to the field.

Also, we will establish ways to collaborate with other scientists and clinicians to develop our scientific results into ways to help patients.

Species and numbers of animals expected to be used

- Mice: 8650
- 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.

Mice and rats will be used as they have a very similar endocrine system to humans. They also become obese and develop diabetes, similarly to humans.

We need to use rodents in our project as metabolic disorders such as obesity and type 2 diabetes are complex diseases. The effects of these diseases occur in numerous organs and systems, which can only be studied in whole animals. Mice and rats closely mimic the human system. Mouse and rat cells are similar to human cells, in the ways they grow, divide, and communicate with each other.

Mice, rats and humans have the same organs and their bodies work in much the same way, and they are also very similar in the way they develop diseases like obesity. They are therefore ideal to help us understand the systems and mechanisms involved in metabolic disorders and to test potential treatments.

While mice and rats are similar in many ways, in some cases rats are a better model for humans than mice. For example, rats and humans both have an increase in their basal metabolic rate after administration of glucagon, which is not seen in mouse. In addition, rats are more suitable for certain studies as their metabolic rate more closely matches that of humans, and their larger body size allows for a greater total volume of blood sampling. However, mouse will be the default organism to use unless literature justifies use of rat.

The work on this licence will use adult mice and rats, as they provide a comparable model to the most common age of diseases such as obesity and type 2 diabetes in humans.

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

The project involves dietary modifications, including changes in nutrients such as feeding animals a high fat diet, mimicking a typical human diet and reduced calorie content, similar to a person going on a weight loss diet. Animals will also be treated with specific experimental agents such as drugs which are being developed to treat disease like obesity and diabetes. Administration of these treatments will follow best practice as recommended by the Laboratory Animal Science Association (LASA) guidelines. In some cases, to test if the potential drug is working and how it is working, animals will have small blood samples taken and might also undergo a scan, to measure physical properties like levels of body fat. Samples from animals' organs and tissues will be taken to study after they have been humanely killed. In studies of energy balance regulation, animals will be caged alone so that the food intake of an individual animal can be monitored accurately.

A typical scenario would be group housed animals placed on a high fat diet for 8 weeks and then be single housed and maintained on high fat diet. Once animals are acclimatised to single housing, the additional environmental enrichment (additional toys, bedding and tubes) and of an adequate body weight (model of obesity) they would enter the 'study

phase'. On study, animals would receive typically 28 peripheral injections over one month (mouse) or two months (rats), with food intake and body weight recorded at least three times a week. Mice would undergo body composition analysis by NMR spectroscopy (EchoMRI) on two occasions, normally at the start and end of treatment period. Mice and rats would have a small blood sample taken at the start of the study and one at the end. Mice would also undergo a glucose tolerance test on one occasion, where they would be given an injection of glucose and 5 small blood samples taken over the next two hours. Over all experiment duration would be 10 weeks in mouse and 14 weeks in rat.

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

Most genetically altered animals used in our project are expected to show only mild differences compared with unaltered animals. Animals subject to genetic models of obesity rarely develop complications which cause adverse effects for the length of this study. However, additional monitoring will be completed to make sure that mobility and access to food are not restricted and they don't develop other complications. Studies for induction of obesity or diabetes are unlikely to result in adverse events within their proposed duration except for consumption of more water and greater volume of urine production. Typically, induction of diabetes by diet results in a mild condition, with no discomfort to the animals or adverse effects. The level of obesity achieved by dietary modification does impact the animal's behaviour or cause adverse effects, for the proposed duration of the studies. Dietary modifications may result in reduced appetite, hunger, and weight loss, which will be carefully monitored.

The substances we are studying have previously been administered to rodents or similar and therefore, combined with data with non-animal studies, will be appropriately characterised prior to administration to animals. While we aim to design safe compounds, one class of compounds we are studying is designed to reduce appetite. The amount of substance to be given to animals will be carefully considered to limit the size of this effect. As the substances are active for less than 12 hours in mouse and rat, any effects they have will be limited to a short time.

Administration of substances, peripheral blood sampling, imaging and telemetry should result in no greater than mild transient discomfort.

To study appetite and energy regulation, animals need to be caged alone to allow accurate measurement of food intake. Single housing rats and mice can have adverse effects on their behaviour, and animals will only be single housed when required. When animals are single housed, additional cage enrichment will be provided, and animals will be closely monitored for signs of behavioural changes. Single housing may be required for up to 50 weeks for males and 24 weeks for females, however, for the majority of animals it will be no more than 45 days.

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

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

The expected severity levels on the animals in this project licence are: 50% mild
50% moderate

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?

Energy regulation involves the interactions of many body organs and systems. Therefore, its study requires the investigation of whole animal physiology. Mice, rats and humans have the same organs and their bodies work in much the same way, and they are also very similar in the way they develop diseases like obesity and diabetes.

While mice and rats are similar in many ways, there are differences which can make one animal a better model for studying what is happen in humans which is why having both available will generate the most useful data for this research. Overall, use of a rodent model allows for the best translation across to human health.

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

When possible and appropriate, substances will be initially characterised using *in vitro* and other non- animal methods.

Why were they not suitable?

In vitro tissue culture based systems alone cannot reliably model the multi organ effects of therapy, nor do they fully reproduce the complexity of the in vivo environment, which critically influences disease presentation as well as therapy response.

Reduction

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

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

The numbers of animals estimated is based on a 5 year programme of work, and covers the research activities of a large and active group. It is estimated that approximately 800 standard mice will be used per year, and the remaining number will be transgenics, the majority of which will be from breeding programmes to generate mice models of interest. It is estimated we will use up to 500 rats per a year. It is expected that more mice than rats will be used, as mice are the default species to use, and rats only used when specific

reasons require.

The natural variation in food intake and body weight between rodents of the same species can necessitate relatively large group sizes to detect changes. However, to ensure the minimum number of animals are used which will provide reliable data, all animal experiments will be carefully planned with advice from the statistical advisors. Statistical tests will be carried out to ensure only the minimum number of animals required for each study is used.

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

We carefully plan all our experiments to minimise the number of animals we need to use and get the most scientific information possible. This includes using tools like the NC3Rs Experimental Design Assistant to plan and organise all parts of the experiment and taking appropriate advice from statisticians and other experts in our organisation.

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

Where practicable, at the end of the studies the maximum number of tissues and measurements will be used from each animal to minimise the number of animals required and maximise the data obtained from each animal.

Resources are being invested into testing and developing new analytical methods, enabling a reduction in the numbers of animals used.

For each anticipated component of work, we will undertake literature searches to ensure that the proposed animal work does not duplicate efforts already undertaken.

Refinement

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

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

Mice and rats will be used in this project. Together these are the most widely used model organisms for studying metabolic processes. The main reason for this is that the systems that regulate these functions are similar in both rodent and human.

The extensive literature in these species means there is no need for numerous preliminary studies. In addition, the experimental models that will be utilised in this study are well understood. Models of obesity and diabetes where animals are given altered diets don’t cause distress or lasting harm, and animals will be killed before they experience any more than moderate adverse effects of the disease. In most cases rats or mice will be used, rather than both, to answer specific research questions.

Genetic factors will be considered along with other factors such as known responses to the substances under investigation to select the most appropriate model.

Genetic models used on this licence have little side effects that means we would not expect animals to experience more than minor and brief discomfort. They will only be utilised where the genetic modification will further our understanding of the mechanism of disease or the mechanism of compound actions.

Animal suffering will be minimised by appropriate use of anaesthesia and analgesia to control pain. Non-invasive methods will used where possible.
Why can't you use animals that are less sentient?

Human metabolism, in particular energy homeostasis is a complex system. While the basic action of appetite control is a fundamental process for life, its complexity has evolved as mammals have evolved. While zebrafish have been of great value in identifying many of the 'components' of energy homeostasis, the complex interactions between these components are not replicated in them. The work to be completed on this animal licence focuses on this interaction and in the development of novel drugs which will modify the system of energy balance. Mice and rats are the most widely used animal model for studying metabolic parameters as systems that regulate these functions are similar across rodents and humans. These are the least sentient animals for this research. Adult animals will be used, since a fully developed metabolic and endocrine system provides the most appropriate model for studying this area, and for development of novel therapeutics for the treatment on disorders which largely occur in adults.

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

We will take a number of steps to minimise harm to the animals. These include extensive investigation of any substance prior to injection in animals and having clear limits on the effects will minimize the chance of adverse effects and suffering.
Anaesthesia and pain-relief medicines will be administered wherever possible and we will conduct experiments on terminally anaesthetised animals whenever appropriate.

Enrichment of the animal's environment will be used routinely, including but not limited to, nesting material, cardboard and plastic tubes, shelters, chew blocks and sticks and aspen balls. Where animals require single housing to enable accurate assessment of food intake, additional enrichment will be used. Working with the NACWO we will continue to trial cage designs which divide large cages into two sections with individual food hopers, to more closely replicate group housing of animals.

As an experienced group we have already refined many aspects of the procedure, such as using narrow gauge needles for substance administration, minimising blood volume required and training rats to move from the balance to their cage, minimising handling. However, I will frequently discuss best practice with the named veterinary surgeon and NACWO.

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

LASA guidelines and ARRIVE guidelines will be followed.

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

We will keep informed about any advances by reading scientific publications and speaking with colleagues within our organisation or/and elsewhere such as scientists who directly involved in the care and welfare of animals. In addition to the NC3Rs website, our institute has a very active 3Rs programme with events circulated to licence holders and advertised on the intranet. Along with personal licence holders, I will attend internal and external meetings and training events to discuss the latest developments in the 3Rs and how to implement them. In general, we will communicate with our NC3RS liaison officer and institute changes whenever we can to improve animal welfare, reduce numbers of animals required and refine the methods. When improvements are suggested, we will discuss these with the NACWO and NVS prior to implementing them to make sure best practice is followed.

13. Pathophysiology and therapy of neuromuscular diseases

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Gene therapy, Muscular dystrophy, Myopathy, Treatment, Glycogen storage disease

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

Examine the disease mechanisms and evaluate treatments for neuromuscular disorders.

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

Why is it important to undertake this work?

Despite knowing the genetic basis of many of the neuromuscular diseases, there are still, commonly, no effective treatments available for these debilitating and often fatal diseases. This is largely due to the substantial challenges associated with developing gene therapies from positive results in cell culture and local delivery to effective systemic treatment.

Unfortunately, assessment of many potential pharmaceutical therapies has been hampered by poor experimental design and in some cases misleading readouts of efficacy. Despite these problems, there is an increasing interest from the industrial sector in bringing treatment for these conditions to clinical trial. However, it is essential that such therapies are based on strong pre-clinical data. The project will be primarily concerned with developing treatments for primary neuromuscular disorders, including Duchennne Muscular dystrophy and glycogen storage disorders.

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

We anticipate that the results of this project will lead to publications in the public domain and will be used to inform clinical trials in humans or in veterinary species. Work towards understanding the mechanisms that lead to the diseases in the first place will provide novel information available to the entire research community to help expedite search for novel treatments.

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

Neuromuscular diseases are an area of high unmet medical need that affect not only the individual patient but also their relatives and others involved in caring for the patient. Often identical diseases occur in animals, and these too, have a paucity of treatment options. Together with the necessary medical support such diseases have a high social and economic impact. Many of these diseases appear spontaneously with no family history, hence they cannot be effectively controlled by genetic counselling. These diseases are debilitating and are often fatal and reducing or stopping the progression of the disease would have a huge impact on the lives of patients and their carers. It is anticipated that the results of this project will be used to support clinical trials as well as to add to our understanding of the pathogenesis of muscular dystrophies and other myopathies of humans and animals. Reagents and techniques developed in this project are also likely to be applicable to other diseases. In some cases, the results might be used to discard therapeutic approaches that are not promising once tested in the whole organism. Both positive and negative results will be published.

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

Much of our work is done in collaboration and we will always share our findings at international and lay meetings (whether positive or negative results obtained). Work will be published in open access peer reviewed journals.

Species and numbers of animals expected to be used

- Mice: 1400

Predicted harms

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

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

We will utilise mouse models of inherited neuromuscular diseases (such as the mdx model of Duchenne Muscular Dystrophy) or genetically modified mice that can be used to inform

mechanisms that cause neuromuscular disease. These animals will generally be bred in house, although they might also be purchased for our studies as juveniles or adults. Animals will be studied at various life stages, including being maintained to adulthood, as we are interested in longevity of certain treatments.

Such animals allow us to evaluate specific treatments (for example gene therapies, or oral therapies) designed for use in related or identical diseases in humans. In many cases, the mouse model will have a mutation in the same gene that is mutated in people with the equivalent human disease.

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

Animals might receive drugs orally (for example in drinking water), or via injection under the skin, into muscle, into a vein or into the abdominal muscle. Some animals might be followed for their exercise ability (on a mouse wheel), or on a mouse treadmill and some animals might be anaesthetised and a procedure performed to test their muscle strength which involves a surgery to expose a nerve and muscle. The nerve can be stimulated electrically (also under anaesthesia) and muscle strength measured with a force transducer. Animals that undergo this procedure are euthanased before they wake up so that they do not feel anything.

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

The animal models used in this research typically have few if any clinical signs associated with their genetic disease (i.e. a non-harmful phenotype). We do not anticipate the work to be associated with pain. Certain neuromuscular disorders are associated with gradual muscle atrophy which causes gradual weight loss (due to loss of muscle mass) which will be monitored. It is highly unlikely that animals will reach a disease stage where their weight loss, or their disease or any treatment will result in any adverse effect that is more than transitory (for example momentary discomfort associated with needle injection).

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

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

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?

In this project we will be examining mechanisms of disease or treatments for

neuromuscular conditions the most important of which is Duchenne Muscular Dystrophy (DMD). DMD is a disease first recognised in young boys caused by mutations in an X-linked gene that is critical for muscle function but that also causes behavioural and cognitive problems. Sufferers are confined to a wheelchair by the age of 12, are effectively paralysed by their 20s and all die in their late 20s or early 30s due to progressive wasting of all muscles including the heart and in particular, respiratory muscle failure. The disease is the most common fatal genetic disorder diagnosed in childhood and has a worldwide prevalence of 1 in 3600-5000 male births. Optimal medical management has improved quality of life and increased lifespan from an original age of death at 16, but can do little to prevent the relentless muscle wasting. We will also be evaluating treatments for a group of conditions known as glycogen storage myopathies which are associated with muscle cell breakdown and weakness in humans and other mammals, including dogs and horses.

The development of treatments for these conditions used cells in culture but mouse models are critical for the next step in the therapeutic pathway. In particular, the mdx mouse (or related mouse models) are excellent biochemical models (lacking the functional protein dystrophin) and they enable testing of therapies designed to increase dystrophin expression in muscle.

In the vast majority of cases the drugs and genetic constructs under test will have been evaluated in cell culture first. However, it is not possible to fully evaluate the treatment effects without testing in an intact whole animal with functional nervous and hormonal regulation of cellular processes and the complex inter-relationship of the muscle/CNS and the vasculature.

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

Cell cultures Organoid cultures Organ on a chip

Why were they not suitable?

For some early experiments we are able to use cultured cells or other approaches to test some hypotheses, but cultured muscle cells, organoids and organ on a chip muscle preparations do not reach the same level of differentiation that is present in mature myofibres in the whole animal and therefore are not useful when evaluating (for example) viral gene therapies as they might not express the relevant cellular receptors for virus update. They are also not associated with the internal milieu of hormones and blood and nerve supply that is fundamental to neuromuscular function. These preparations are also not suitable for evaluation of oral or sytemic dosing regimens.

Reduction

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

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

Determining the correct number of animals to use in an experiment is a critical issue: we will ensure that sufficient numbers of animals are used to generate a robust, statistically

significant result that is of biological/disease importance. However, we will also ensure that we do not use any more animals that are necessary to demonstrate this effect. These numbers can be determined, by prior understanding of the effect that is being looked for and the variation within the animal population.

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

We examine prior published research literature to allow us to be informed on extent of variation within the animal population and the effect size that we are trying to or can reasonably expect to achieve.

This effect size must be of biological or disease importance for us to proceed. The NC3Rs Experimental Design Assistant will be used for algorithm-generated feedback on adjustments that could be made (such as identifying potential sources of bias/nuisance variables) and, where appropriate in representing experimental design visually for group or external discussion. We utilise other online resources (such as GLIMMPSE) to ensure that numbers of animals used will maximise the chance of a positive outcome, when using repeated measurements, to increase the power of our statistical comparisons. Where possible, we use stored tissues from previous studies, to reduce the number of animals required. Experiments are typically conducted according to ARRIVE (2.0) 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 only breed animals for specific experiments or to maintain a colony. Wherever possible, surplus tissue from animals will be shared with other researchers.

In novel experiments, we will conduct pilot studies, where appropriate, to help inform our future 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.

We will use murine models and in particular, models that have a mutation or are transgenic to make them suitable for investigation of neuromuscular disease.

The genetic mutations or genetically modifications we use, are not knowingly associated with any discomfort to the animal or any suffering.

In some animals we will study muscle physiology with the animal under general anaesthesia, from which the animal will not be allowed to wake up, such that no pain will be felt.

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

Mouse models allow us to examine responses to neuromuscular diseases, which are crucial when evaluating treatments and disease mechanisms to humans or other mammals. Mammalian models are needed accurately to assess the side effects, safety and efficacy of treatments.
Often, treatments are administered to these animals at young age, but then they are studied towards adulthood so that long-term safety and efficacy can be assessed - the principal reason these animals are being used.

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

Animals will be closely monitored for signs of suffering or welfare problems, however we do not anticipate welfare costs as the phenotypes we are studying are not painful or associated with welfare issues in mice and the animals are not undergoing surgeries from which they recover. We will not perform procedures that are associated with expected pain, other than momentary discomfort.

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

We will follow guidance generated by the NC3Rs in terms of husbandry. We follow LASA guidelines whenever appropriate for administration routes and blood sampling protocols.

In addition, work conducted within our facility is performed with attention to the Culture of Care, promoted by the PREPARE guidelines to which we espouse in ongoing work and in particular, when planning and preparing future projects.

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

We will conduct regular 3Rs assessments for our work and utilise online tools and information and advice from the NC3Rs including their Resource Library and Resource topics. We will communicate with our NC3RS liaison officer and institute changes whenever we can to improve animal welfare, reduce numbers of animals required and refine the methods.

14. Social influences on foraging and food caching in grey squirrels

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Social Networks, Decision-making, Machine learning, Automated tracking, Foraging

Animal types	Life stages
Grey squirrels	adult, pregnant, aged

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim of this project is to use automated tracking technology and machine learning to understand how grey squirrels manage their highly flexible social relationships and to test how decisions made by individuals about foraging for food and storing it for later use are influenced by this social environment. To achieve this, I will develop a method for automated detection of decision-making in a wild population of squirrels, and combine this with technological methods that allow me to measure social relationships and home ranges.

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

Why is it important to undertake this work?

Grey squirrels are a very successful invasive species that has proven to be highly resistant to control via culling. They are striking in the speed at which they repopulate areas that have been cleared and this may be due to their highly flexible social system which makes them able to tolerate and thrive at very high population densities. An understanding of how individual grey squirrels manage complex social interactions may help to inform new

methods of pest control.

From a basic science point of view, it is also important to understand how an individual manages its' own social position and how this management relates to decisions that are crucial for survival.

Decisions are a crucial part of everyday life and they are made in a variety of situations which may influence the outcome. Social situations are particularly relevant to decision-making because often the outcome is dependent on what others are doing at the same time or might do in future. The study of decision-making is often done on isolated individuals in artificial laboratory studies, so it is important to see how and whether our understanding of decision-making stands up in real life situations. My study system, focussing on wild animals, gives a unique opportunity to look at social influences in decision- making in a model system that will then inform research more widely. Wild squirrels make decisions about whether to eat or bury nuts for future use, akin to investing in the future. Investment decisions are influenced by economic considerations such as the value of the food, generally availability, current need and likelihood of future recovery, but social considerations are likely also important more generally because they can determine future availability in terms of whether or not food is stolen by onlookers and competitors, but also more specifically in terms of social position and social standing.

We are interested in how nuanced social interactions influence foraging decisions, for instance individuals with stronger social bonds may have better access to the stored food of others and social position may well influence risk of loss of stored food. Understanding the importance of social factors in investment decisions will add to the complexity of our knowledge and will provide a gateway to understand the general principles of social influences on economic decision-making across the animal kingdom.

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

By using a combination of recent technological innovations, this project will allow me to investigate how the social environment influences decisions about saving for the future (investment) at a level of detail that has previously not been possible. This will contribute to our current understanding of decision- making in dynamic social environments. The project will also contribute to knowledge of how the combined use of sophisticated technology can be used to create high-quality detailed datasets that allow investigation of novel questions.

Knowledge about grey squirrel's social interactions and their interaction with foraging decisions will provide a valuable contribution to DEFRA's efforts to control grey squirrel numbers in the UK. Underpinning control methods with a detailed understanding of behavioural factors could help to provide the missing link in successful culling programmes which are widely used for population control and management.

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

This project will provide basic science benefits by giving a broader understanding of the influence of the social environment on decisions about saving for the future in a model species. Given that decision making is a fundamental aspect of life, this knowledge is applicable to a wide variety of fields such as animal behaviour, psychology and economics. More specifically, it will provide information about how animals that store food for future use, and rely on these food stores, may be impacted by their social environment.

This project will help to formulate more general principles about social influences on economic decisions that could be applied to human saving behaviour by providing knowledge of how the social environments affects a vital behaviour seen across a range of species.

An additional longer term benefit of the project will be to pioneer a new method which will allow a link between researchers in the field of social networks and cognitive and behavioural sciences. Cross discipline collaborative work will foster a deeper understanding of the role that sociality plays in the lives of animals and humans.

Finally, this project may benefit DEFRA's efforts to control invasive grey squirrel populations in the UK by providing a level of detail about their social interactions and minute-by-minute foraging decisions that could be used to refine culling protocols and targeted areas of population control and management.

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

In order to maximise the outputs of this work we will publish the methods and results in peer-reviewed journals, present the findings at international conferences, work with the press office to create press releases and we will participate in events which are designed to increase public understanding of science such as Soapbox Science events locally. We will liaise with the APHA's wildlife team and the UK Squirrel Accord research committee to disseminate our results and discuss applications to culling programmes.

This work will also foster collaborations between researchers in different fields and PhD students involved in the project will learn new skill sets which will improve their employability and knowledge and allow for them to develop as independent researchers with potential to lead to increase basic science related to knowledge and conservation in the field.

Species and numbers of animals expected to be used

- 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 are using grey squirrels because they are a model species for addressing the aims of our research. They readily cache food and they do so in a social setting that is easily accessible for data collection.

The grey squirrels on campus, known as the Exeter Squirrel Project, have been the subject of behavioural studies for 20 years and, as such, are an ideal subject for this work. They are known individuals, individually marked, tolerant of human presence and readily engage in experimental and observational set-ups so they are easy to watch and collecting data from them is not difficult, making them an excellent wild species for looking at real-life decision-making. Using wild animals in a 'living laboratory' model is supported by the University as a move away from working on laboratory held animals, who often

show abnormal responses to captivity. This is particularly important for work where behavioural questions are the focus of research, and there are no known purpose-bred species that could be held in a lab to answer these questions because of the extensive caching of individual items done by the squirrels.

Grey squirrels are notable for their incredibly flexible social system that allows them to tolerate very high population density. This level of social flexibility is unusual in animals and may well contribute to their success as an invasive species. Understanding the nuances of their social flexibility and the effects this has on their food storage strategies could make a valuable contribution to effective control measures of this species, which has been surprisingly resilient against standard control measures.
This work, along with other projects using the same population of grey squirrels, has been approved by Natural England and granted a Non-Native Release license in order to facilitate marking of individuals. There is scientific value to keeping the animals alive as they are part of a long-term study that will aid our understanding of behavioural contributions to their survival and fitness. Using wild squirrels allows us to measure natural social interactions and decision-making in a complex social network, which we will be in a position to map out at a level of detail no one has yet been able to obtain. Adult squirrels (who have reached full size) will be used for collaring in order to minimise the effects of the collars on their welfare and behaviour since the collars will be proportionally lighter compared to their overall body weight and they will not grow during the course of the study, meaning that the fit of the collars will not change over time.

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

A typical squirrel would enter a trap set in sheltered, wooded areas of campus. Its health will be assessed and if healthy and fit, will enter a handling cone where it will be restrained for around 10 minutes. It will be sexed, weighed, and the length of its back foot measured. It will have an identification chip placed under the skin if it does not have one present already and, on first capture, it will have a few hairs plucked for DNA analysis. It may also have dye placed onto its coat for additional identification. A tracking collar will be placed around its neck, after which it will be released back into the wild.

Once collared, the squirrel will be tracked in order to record its social interactions and activities for a few weeks before it is re-trapped and the collar removed. The squirrel will be recaptured at 2-3 time points for recollaring in order to look at seasonal changes in activity in the same squirrel.

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

We expect minimal impacts and no adverse effects during this project. Collars may cause a short period (a couple of hours) of stress from being fitted, which can cause the squirrels to scratch at them. Collars will be worn for 2-6 weeks and then removed when the animal is re-trapped. A very small number may develop skin irritation from the collar. We have consulted the wildlife research team at the APHA and a team of researchers in North America to provide help and advice, and they are confident that our planned methods are minimally invasive and that harms will be low. APHA has had retrapping success of 100% at some times of year, and we are confident we will recapture the squirrels with a high success rate.

Expected severity categories and the 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 animals will undergo procedures that are classified as mild.

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

- Set free
- Kept alive

Replacement

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

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

There is no alternative to using animals for this project if we are to fully understand social effects on behaviour in wild animals and, more specifically in grey squirrels. Research on animals is essential to examine and record social network position and real-life decision-making at the level of detail we will be able to achieve, which has not been done before in grey squirrels. However, we are using wild animals in a 'living laboratory' model, which is a move away from working on laboratory held animals, who often show abnormal responses to captivity.

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

There are no suitable non-animal alternatives for this project.

Mathematical models and simulations can be effective ways to examine the influence of social position on decision-making, but we need data in order to test models and we need to be able to test the predictions of any model in the wild to ensure the models are realistic. To understand how social standing influences economic decisions in nature, it is therefore critical to obtain data to determine (a) the structure of social networks in grey squirrels and (b) the decisions that they make about storing food in the wild. In this project such data, obtained through tracking the squirrels with collars will be analysed using sophisticated statistical techniques to generate robust results.

Why were they not suitable?

Mathematical models and simulations are only useful in conjunction with real-life data to test whether their predictions are realistic, they cannot be used alone to investigate this research question.

Research on wild animals is essential to understand social influences on 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?

All proposed protocols are based on previous successful research by the applicant and others. The applicant is experienced in complex multifactorial analyses and is in close contact with statisticians. Generalised Linear Mixed Models will enable analysis of normally and non-normally distributed data, controlling for repeated measures where necessary, ensuring the greatest possible statistical power for any given sample size.

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

Observations have minimal impact on the squirrels compared to trapping. Therefore, we will carry out multiple observational procedures on the same marked individuals to ensure we get high quality data that can be used for several analyses while reducing the number of animals that need to be trapped.

To understand the complexities of social life in squirrels, it is critical to observe and record social interactions across contexts. To establish this, it is necessary to fit individuals with tracking collars so as to monitor their movements. The number of tagged individuals will be kept to the minimum (N<80 squirrels) necessary to determine association networks. Tags will also contain accelerometers so that machine learning can determine behavioural categories, allowing us a detailed picture of the interplay of sociality and behavioural decisions. Even in the unlikely event that up to 20% of the tags fail to record, a sample size of 80 individuals will provide a robust number of social interactions for analyses to test the prediction that social network position influences behavioural decisions across seasons.

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 observational data from previous work addressing a different question which suggests that we can collect robust social and behavioural information from approximately 60 squirrels, which allows for some tag failure if we aim to tag 80 individuals. The tags will allow for much more detailed data collection than an observational study allows and will give us insight into the squirrels' behaviour when they are out of sight, as animals go out of sight in undergrowth, bushes and tree canopy regularly and it is impossible to get accurate social network data when so many animals are present but not visible at any point in time.

Refinement

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

Which animal models and methods will you use during this project? Explain why

these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Grey squirrels will be used for this project. Wild adult grey squirrels will be caught, PIT tagged, collars placed on them and then released with recapture when the battery dies approximately 2-4 weeks later. Collars have the capability to transmit data in real time so we can detect battery death when data stops transmitting.

The capture method uses live-traps specifically designed for use on squirrels. The traps are baited well with peanut butter, placed in sheltered locations and checked at intervals of less than 6 hours. This ensures that the captured animals have food, space and shelter while in the traps and minimises any distress.

Squirrels will leave the traps and enter a wire-mesh cone for gentle restraint while the tags are fitted. The cone reduces the need for handling, which can be stressful for wild animals, because it fits on the end of the traps and the squirrels run straight into the cone, which has been designed for use on adult squirrels. Once PIT tags have been injected and collars fitted (a process that should take less than 10 minutes, squirrels will be released directly from the cone at the point of capture. When the battery dies, squirrels will be live-trapped and collars will be removed using the same trap and cone system. We have discussed the use of quick-release collars with a number of squirrel researchers at the APHA and in North America (from the Kluane Squirrel Project) and they are not feasible for this species – any weak point that would allow slow decay and drop off would be immediately chewed off by other squirrels sharing a drey. The alternative is to load a small explosive that breaks the collar remotely, but squirrels are too small for this to be a safe alternative for use here. Our recapture rates using normal trapping procedures, i.e. making no concerted effort to re-trap particular individuals and including individuals caught on the last day of the trapping session, are 60%, and we are confident that by only collaring individuals we have caught before and by target trapping by focussing on core home range areas and around dreys (which we will know from the remote data collected from the collars) will increase recapture rate where necessary. A current APHA project has a retrapping rate of 100%, so we know it is possible. Our consultations have also assured us that previous squirrel research projects have assessed that safety issues for the squirrels using collars similar to ours are negligible, at less than 1%.

In order to minimise pain, suffering and distress via the cost of carrying a collar, collars will be less than 5% of the squirrels' body weight. Tracking squirrels using collars is a common method used in research projects with very little harm to the animals. The collars are custom made and follow a design specifically made for use on squirrels, which ensures a good fit. Collars are made of brass so that they cannot chew through them, and coated with smooth plastic in order to reduce rubbing/irritation and fur pulling. We will observe released squirrels directly and remotely, and so we can note any accidents or distress related to the collars and we can target trap for removal if necessary. Protocols are in place for detailed monitoring of behaviour and morphometrics post-release. At any stage, if deleterious effects are noted on any squirrel carrying a tracking collar, every attempt will be made to capture the relevant individual and remove it. The applicant has received approval for trapping and releasing grey squirrels on the POLE from Natural England.

This protocol will be repeated at intervals of 4-8 months for the course of the project such that some individuals will be under continued use, as well as tracking new recruits.

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

Grey squirrels are used as they are free-living and they regularly make economic decisions when storing food for the winter months. They are an ideal species for investigating social influences on the foraging and food storage decisions that they make, and so it is crucial to use them for this work. In addition to this, using less sentient animals would not give us any information that might be useful for culling grey squirrels in particular. We will avoid using juvenile squirrels in this study as they are smaller and so the tags would be proportionally heavier for them. There are no less sentient species that store food for future use, so it would not be possible to use a less sentient species here.

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

Animal suffering will be minimised by ensuring that all staff are trained and competent in the attachment and monitoring of collars on squirrels. We have a health assessment checklist designed in conjunction with a veterinarian and a NACWO which we will implement prior to use and release to ensure that only healthy animals are used with the greatest chance of successful release into the wild. In addition to this we will carry out daily visual checks where possible as well as tracking the animals with the data from their collars to note any behavioural changes. Collars will be removed from the animals at the end of battery life, which is estimated at 2-4 weeks, by re-trapping the squirrels.
Only animals who enter traps will be tagged, which self-selects for bolder individuals who may be more robust to the procedures.

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

I will follow published Home Office Guidelines, specifically the document "Animals (Scientific Procedures) Act 1986: Working with animals taken from the wild"

I will also follow The Association for the Study of Animal Behaviour's Ethical Committee's "Guidelines for the ethical treatment of nonhuman animals in behavioural research and teaching" published in Animal Behaviour (2023)volume 193

Finally, I will implement recommendations from the STRANGE framework in order to ensure the data from this study are reproducible.

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

I will keep up to date about advances in the 3Rs through regular lab meetings, discussions with our NACWO(s), NVS and NIO and our NC3R Regional Programme Manager. I will keep up to date with the literature in the field and maintain communication with other wild animal tracking researchers and attending relevant conferences on refinement of animal tracking techniques. Where feasible, I will implement any advances during the course of the project.

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

Key words

Inflammatory bowel disease, Cancer, Obesity, Autoimmunity, Lymphocytes

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 understand the interactions of innate and adaptive immune cells that cause autoimmunity, obesity and responses to 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?

To discover basic mechanisms of diseases in order to design new therapeutics and diagnostics.

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

New information, scientific publications, new biomarkers and therapeutics

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

In the short term, these outputs will contribute to better understanding of disease development and progression. In the medium to long term, patients with inflammatory bowel disease, obesity and cancer will benefit from new treatments and diagnostics.

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

National and international collaborations, conferences and publications

Species and numbers of animals expected to be used

- Mice: We estimate ~5500 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.

Complete immune system responses cannot be accurately modelled *in vitro* and addressing complex immune interactions requires the use of experimental animals. Mice are the preferred experimental animal due to their anatomical, physiological and genetic similarity to humans as well as the practical advantages of their small size, ease of maintenance, short life cycle and abundant available genetic resources. We will be using exclusively adult animals for experimental work in order to work with a fully formed and functional immune system.

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

Typically genetically altered mice will be bred specifically and placed onto a particular experimental protocol. Often mice will receive injections of substances to influence the immune system or the genetic status of the mouse or other immune interventions before proceeding to the main step of the protocol , generally the induction of an immune condition such as colitis or tumour growth or a physiological condition with immune relevance such as heart remodelling Sometimes this will involve surgical techniques such as implantation of gut tissue or heart transplant. Endoscopy may be performed to investigate development and progression of colitis. The mouse diet may also be modified.

Samples and readings may be taken throughout the experiments, these might be blood samples, physiological readings, imaging , metabolic testing or similar. Animals are generally limited to a single procedure and the duration of experiments is kept as short as possible to obtain the necessary data, mostly being a duration of weeks extending out to months only in rare cases.

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

Many mice falling under the Mild (or below) category will experience only transitory and short lived pain (such as from an injection or from ear marking) and possibly no adverse effects at all. Mice falling under the moderate category may experience pain and weight loss , for example while undergoing colitis protocols , tumour growth under tumour protocols or potentially pain under surgical protocols (i.e heart graft, implantation of Osmotic minipumps or telemeters). Mice undergoing Immune depletion may be treated with radiation leading to sickness leading to lethargy, inappetence, diarrhoea and weight loss. Adverse effects are strictly controlled for all protocols with humane endpoints established to minimise suffering at all stages , duration of any experimental protocols likely to cause adverse effects is kept to the minimum required for experimental validity.

Expected severity categories and the 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 animals to fall mostly under mild severity category or below with a smaller number falling under the moderate category. Most animals bred under standard breeding protocols will be mild or below as will any animals used exclusively for tissue or used under certain protocols (Dietary manipulation, infection). Animals used under other protocols (i.e Colitis, tumour growth) will generally fall under the Moderate category . We estimate 60-70% of animals used will fall under the Mild category or below with the remainder being 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?

There are currently no lab techniques that can accurately simulate the entire immune system and this means that animals are required for this kind of research. Replacement opportunities such as organoid/3D organ culture have been investigated by the lab and have been used to simulate aspects of the gut immune environment and may be used to replace animals under some circumstances.
Further replacement technologies such as organ on a chip (chips with engineered or natural tissue designed to mimic animal physiology) are becoming increasingly advanced and may in the future allow more complete simulation of the immune system.

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

Our group is active in attempting to use lab based tests to simulate various individual

aspects of the immune system where possible in order to try and minimise the requirement to use animals. We make use of human tissue and cell lines where possible and appropriate. The lab has made use of and developed protocols for organoid and 3d cell culture that allow simulation of some aspects of the gut immune system allowing potential replacement of animals for some experimental work.

Why were they not suitable?

It is not currently possible to reproduce the complexity of a complete immune system with laboratory cell line based approaches or with tissue in vitro (either human or mouse). Human material is also much less readily available and does not have the genetic flexibility of 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?

Based on usage of current protocols that we propose to continue using and also by experience of the models and techniques used. We have a good understanding of the type and magnitude of the results we expect and therefore the size of experimental group and number of repeats needed to obtain biologically significant results.

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

We have utilised the NC3R's Experimental Design Assistant as well as only performing experiments that we know from experience give the clearest results, thereby reducing the need for experimental repeats and reducing the number of animals required.

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

We are committed to maximising the amount of experimental information we obtain from each individual experimental animal, enabling us to hopefully minimise usage. We routinely use single strains of mouse in multiple research areas, use multiple organs from the same mouse and share tissue across multiple users and experiments wherever possible in order to keep animal usage down. We are also trying to minimise the amount mice we breed for each research area by using efficient breeding and cryopreserving lines that are no longer in immediate use, thus reducing the need to breed animals purely to maintain lines (i.e "tick-over" colonies). Mouse numbers can be viewed in real time with use of online stock management allowing better control of numbers.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative

care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

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

We will be breeding genetically altered mice for use on the project and breeding protocols do not cause any lasting distress or suffering to animals with any discomfort being associated with identification marking and being strictly temporary. Experimental protocols are primarily models of human disease (colitis, cancer, heart disease, infection etc) and are planned as to cause the minimum pain, suffering and distress while still delivering experimental data. Each protocol has strict humane endpoints and monitoring to ensure that animals do not suffer unduly.

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

Mice are the preferred species for immunological research , suffering from many of the same diseases as humans for the same genetic reasons. They are also easily genetically manipulated allowing direct investigation of genes of interest. Mice also available inbred as to be genetically near identical which produces less variation in results and more accurate and useful disease data. From a practical standpoint they are also small and easy to house with a short life span and fast life cycle making them a costs effective research tool. Mice are one of the most common experimental animals and are generally considered less sentient than other species that may be used for research (i.e dogs, cats, primates). Adult animals are used as the nature of the protocols generally involve a period of progression over days/weeks and so use of immature life stages is not possible nor is use of animals that have been terminally anaesthetised.

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

All procedures include monitoring of animal health and condition and post operative care and pain management we believe appropriate but we will make changes as required should any regime prove to be inadequate or as suggested by animal welfare staff. Any mice that are immunosupressed due to genetic status or due to experimental treatment will be maintained in a barrier environment to limit exposure to infection. We have previously made adjustments to wording of the animal licence as well as to procedures based on observation and advice and we would see this as an ongoing process.

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

We will look to follow any published refinement guidance from bodies such as NC3Rs that is relevant to our protocols as well as following and implementing any changes suggested by animal welfare staff (NACWO, NTCO etc) or communicated to us through official channels (The Home Office). Examples of best practice guidelines informing animal use would be Guidelines for the welfare and use of animals in cancer research | British Journal of Cancer, Blood sampling:Mouse (NC3R) and mouse handling (NC3R).

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

We will follow advice from animal welfare staff at our institution regarding any 3Rs advances or improvements to techniques and ways of working. We are in regular contact with the NACWO (Named animal care and welfare officer) and NTCO/NIO (Named training and competency officer/Named information officer) and regularly pass detailed experimental plans to them for approval and comments. Animal users are encouraged to take advantage of the specific 3Rs information available on establishment webpages and also take note of communication, webinars and training offered by NC3Rs (National centre for the replacement, refinement and reduction of animals in research).

16. Sensory processing in the mammalian 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

Hearing loss, Vestibular dysfunctions, Sensory systems, Ageing, Brain

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim of this proposal is to investigate how we develop our ability to process sensory information from the outside world, primarily auditory stimuli, and to understand the mechanisms leading to sensory function and dysfunction. We want to define the critical physiological and morphological steps required for the maturation and function of the auditory (hearing) and vestibular (balance) systems. We will also investigate how auditory and vestibular information gathered by the peripheral sensory organs are processed by the central nervous system and integrate with visual inputs to help the brain deciding the best action to take (e.g., either escape or stay still in the presence of a danger situation). Finally, we will investigate whether the progressive loss of sensory inputs with age, especially age-related hearing loss, affects the onset and progression of neurodegenerative diseases such as Alzheimer's.

The discovery-based research part of this proposal will be fundamental to identify the mechanisms leading to different forms of hearing loss and vestibular dysfunctions. These finding will be used for the translational component of this proposal, the aim of which is to develop potential therapeutic intervention to ameliorate or cure hearing loss and vestibular 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?

The sensory systems are key to our daily life, including the auditory and the vestibular systems. For example, the auditory system allows us to perceive sound in all of its different forms, from noises to speech and music. The sensitivity of this system is remarkable, allowing us for example, to detect sounds as quiet as the drop of a pin or as loud as an explosion or an airplane taking off. It is also key for survival in many animal species, as its speed in processing information is unparalleled among sensory systems. This is the reason why time-critical sports such as sprint races start with a gunshot and not, for example, with a flash of light.

Despite the importance of the sensory systems, we still have a poor understanding on how they achieve their unparalleled performances. How does the brain develop its ability to extract information from sounds, movement of the head in space and light? How does it combine this information from the different sensory modalities to make decisions to drive behaviour? These are critical questions in our quest to understand how the brain works, as well as to understand the physiological basis of sensory disorders.

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

The main outputs of the proposed project are publications in peer-reviewed journals and the identification of potential therapeutic targets to slow down or even prevent hearing loss and balance disorders. In addition, we use our research to deliver outreach activities in public events and school visits.

The translational part of the project will also lead to potential proofs of principles for the development of therapeutic interventions for the treatment of different forms of hearing loss and vestibular dysfunctions in humans.

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

Hearing is key sense that, in addition the other sensory inputs such as balance and vision, allow humans to interact with the world round us. Acoustic stimuli are detected by extremely sensitive sensory cells named hair cells that are located inside the ear. When hair cells are damaged, they cannot be replaced, and this is the reason why people lose the sense of hearing, especially with age.

Hearing loss is the most common sensory deficit and one of the most prevalent chronic deficits. About 12 million people in the UK and ~500 million worldwide have disabling hearing loss (>1 billion people expected by 2050) [WHO 2021]. Hearing loss excludes people from basic day-to-day communication, which is associated with significant psychological and medical morbidity, including social isolation and depression. Hearing loss in mid-life is the largest modifiable risk factor for dementia. The number of people affected by hearing loss is increasing in the elderly, affecting their quality of life. Current evidence has also highlighted the association between hearing loss and an increased risk of age- related cognitive decline, incidence of dementia and Alzheimer’s disease.

Currently, our ability to develop new effective treatments for auditory disorders is limited because very little is known about the underlying molecular and cellular mechanisms leading to their dysfunction.

In the short term, the work highlighted in this proposal will not only identify the mechanisms underpinning one of the most common sensory dysfunctions, but also its possible association with cognitive decline. The work will also develop therapeutical approaches for these diseases.

In the long term, this work will allow to translate these therapeutic interventions to humans, with the aim of ameliorating or preventing different forms of hearing loss.

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

The work will deliver several scientific publications of the mechanisms used by the auditory and vestibular system to develop and function, including studies showing the results obtained from the gene-base therapeutic approach. Dissemination of the data will also be maximised via invited seminars and scientific conferences around the world. The findings will also be disseminated to the public via the several outreach activities performed by the hearing research group.

Species and numbers of animals expected to be used

- Mice: 60000
- Gerbils: 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 use of animals is absolutely essential for this project since at present it is the only means of obtaining any fundamental information about the physiological and morphological properties of cells and neural networks responsible for sensory perception.

The mouse and gerbils are currently the least sentient animal model to study sensory processing and the mechanisms leading to sensory function and dysfunction in vivo.

This is not only dictated by the several similarities of these sensory systems among mammals, at both molecular and cellular level, but also due to the ability to generate animals (primarily mice) with similar sensory defects as those present in humans. Additionally, the gerbil cochlea covers a very similar frequency range to that of humans (less optimal in mice), making this mammal an ideal model to study sound processing. As such, the findings obtained from mice and gerbils will be used to further our understanding of sensory function and dysfunction in humans.

Because we are interested in studying how the sensory systems become progressively specialized to detect environmental stimuli, we will use mice and gerbils at all stages of development: from the day of birth up to adult and aged animals. From this work we will be

able to uncover the mechanisms leading to the development, function and ageing of cells, tissues, and whole organs.

Finally, because several sensory dysfunctions in humans are well-replicated in transgenic mice, we will use them to devise therapeutic strategies to target sensory disorders.

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

Several of the proposed experiments will be done on tissue harvested from both mice and gerbils, which will be killed by a schedule 1 method (ex vivo experiments in Objective 2, Protocols 1-3).

Some transgenic/mutant mice may be treated with substances to induce or repress gene expression by injection or in their drinking water (doxycycline, tamoxifen, CNO) prior performing experiments (Protocols 4-8). The application of these substances will be done without anaesthesia and could last several days.

Animals will be anaesthetised (injection or inhalation) to perform procedures such as: delivering locally AAVs for gene therapy; delivering compounds to induce hearing loss and vestibular dysfunctions; performing in vivo behavioural assessment (e.g., ABR, DPOARs and VsEPs) (Protocols 4-8). These animals may be recovered for further tests or killed by a schedule 1 method or a non-schedule 1 method under anaesthesia. Anaesthetised animals are placed in a warm pad to maintain their body temperature throughout the procedure. Experiments normally last no more than 3 hours.

Some animals will be exposed to loud sound noise to mimic its effect on the progression of age-related hearing loss, which is a phenomenon observed in humans (Protocols 4-8). This procedure is performed in the absence of anaesthesia and normally last no more than 5 hours.

When animals are be used to perform ex vivo experiments from the brainstem (Protocol 6), animals will be decapitated since schedule 1 methods and anaesthesia prevent or compromise the project. This is a very rapid method. See below for justification linked to decapitation.

Experiments involving extensive surgery will require mice and gerbils to undergo deep or non-recovery anaesthesia by injection or inhalation. Longitudinal investigations of brain function will be done from awake animals (Protocols 4-8). Experiments normally last no more than 5 hours.

When studying the progressive decline in sensory processing and cognitive decline with age, some animals will be maintained up to about 24 months (Protocols 4-8). Currently, we mainly use the following wild-type mice: CBA/CaJ, C3H and C57BL/6.

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

General impact/adverse effects:

Several of the transgenic/mutant mice used in this project already have hearing and/or vestibular dysfunctions, such as those present in people. The majority of these mice will experience no problems apart being deaf, which is not a life-threatening condition (sub-

threshold or mild severity). A small proportion of mice may experience, in addition of being deaf, different degree of hyperactivity, dizziness and circling behaviour (mild severity). Very rarely, mice may experience more severe phenotypes (moderate), such as difficulties with drinking and eating; in this case animals will be feed with mash and if not able to recover, they will be humanely killed using a schedule 1 method.

Procedure impact/adverse effects:

The work performed on ex vivo tissue will require the killing of the animals using of a schedule 1 method or a non-schedule 1 method under anaesthesia. Therefore, animals will not experience any adverse effects.

Noise exposure, which will be performed in the absence of anaesthesia, is quite well tolerated by mice and gerbils and the only adverse effect noted to date is that they exhibit a “freeze” response at the start of the loud sound (but adapt very quickly).

Some experiments will be done under non-recovery anaesthesia, and as such will never experience any adverse effects.

Procedures involving recovery from anaesthesia, animals may experience post-operational local pain for a few hours and possible infections. Both these possible problems are addressed in the management of the adverse effects.

Animals undergoing decapitation are likely to experience some initial level of stress in preparation for the procedure, which is extremely rapid to execute.

Some animals undergoing systemic injection of tamoxifen, which is used to regulate gene expression, may experience an initial weight loss.

Animal undergoing brain surgery to perform in vivo imaging during ageing may develop infections, which are addressed in the management of the adverse effects.

Aged animals up to about 24 months may display signs of benign morbidity that can be very variable between mouse strains. We have been monitoring ageing mice for over 5 years, and the most common side effects were weight loss and skin rashes. However, only a very few mice reached the humane endpoint, which was for skin rashes not healing following treatment.

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

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

The majority of animals should experience no more than mild severity or non-recovery (typically 70- 80%) with a subset of animals experiencing no more than moderate severity (20-30%).

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?

Understanding the basic biology and diseases in living organisms is a major challenge for scientists. However, complex biological systems that span from the periphery to the central nervous system cannot be replicated in non-living animals (at least at the moment). This is additionally complicated by the fact that the sensory system, such as the auditory and vestibular, are deciphering and integrating several environmental signals. As such, scientists are dealing with a twofold experimental design: how to represent with high fidelity such intricate sensory systems and how to combine this with the large variability and dynamic range of environmental cues. Currently, we do not have the answers for all these problems, which is why we need to use living organisms to gain the required information to develop more bench-based and theoretical approaches.

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

Theoretical models represent an additional approach that can be used to address aspects of our research questions. Currently, we are developing projects that will use this modelling approach to predict animal behaviour. However, models are only as accurate as the information used to inform these models. As such, a meaningful model can only be delivered if we use real measurable data from the biological process under investigation. Currently this can only be obtained from in vivo animal experiments. Therefore, theoretical models and in vivo animal experiments must be used in conjunction, at least for now.

Organotypic cultures have been used for some experimental work on mammals related to this field, including testing the susceptibility of the hair cells to several ototoxic compounds. However, we still rely on in vivo animal experiments to fully understand organ development and function, although potentially the testing of compounds ex vivo or in vitro could reduce the number of animals used.

Why were they not suitable?

Organotypic cultures are not suitable because they lack the complex architecture of the nervous system, which prevents signal processing and sensory integration. Theoretical methods are not suitable because they can only be used to provide ideas on how a system may work, but they cannot address the problem directly. Although animal models are still the only means to understand the intricate mechanisms implicated in the development and synaptic transmission in the nervous system, we are currently working with colleagues to build a computational model of sensory function using the data collected by our 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?

The large number of animals (especially mice) is primarily dictated by the complex breeding strategy of all our colonies, most of them being double or triple mutant mice. The number of animals used is estimated based on previous work done in our lab, which has about 15 years of experience in working with these mammals under three previous 5-year licenses. Moreover, we use the expertise in our animal facility. This has always ensured that the use of animals is kept to a minimum. For complex electrophysiological and imaging functional studies, we will require at least 10 animals per experiments to reach a meaningful statistical analysis. The total number vary a lot depending on how many experimental protocols we use. Also, the total number listed above also includes the future breeding stock required to maintain the complex colonies.

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

Several of the approaches used in this proposal (e.g. AAV injection and in vivo surgery), and the selection of the most appropriate anaesthetic for the different procedures, were developed in my group over the last several years. This will allow us to drastically reduce the number of animals used under this project.

The number of animals used will be kept to a minimum by making sure that all experiments are meticulously designed and by ensuring a high level of training for the staff involved in the various procedures. We also use a rigorous statistical approach.
To reduce animal surplus, we follow the very strict breeding guidelines from our animal facility

In order to decide the sample size needed for the experiments, we use the statistical inference technique suggested in the NC3Rs website.

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

To help optimising the number of animals raised, I follow strict breeding recommendation on transgenic mice, such as only raising those that are of the correct genotype and required for experiments and maintain the correct number of breeding pairs. We will also use pilot studies where applicable to determine baseline parameters.

All personnel involved in the project undergo a compulsory training in all procedures used prior working with animals, which is designed to keep the number of animals required to a minimum.

Moreover, we will gradually reduce the number of transgenic mice imported and breed since we are in the process of establishing the AAV system to deliver the different reporter dyes for imaging (e.g., GCaMP) in cells.

Refinement

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

procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

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

The use of the mice and gerbils as models demonstrates the use of a species of a lower neurological sensitivity compared to primates. Rodents are the most refined model to use for our studies as the similarity between humans and rodents both at the genetic level and also at the level of ear structure/function mean that experiments performed on these animals can be used to understand human genes and ear structure/function.

The large majority of the procedures are designed to test physiological aspects of the sensory systems using imaging and electrophysiology methods, which are non-invasive techniques and does not require any surgical operation. However, the auditory and vestibular system are well embedded in the skull, which is the reason why surgery is sometime required to reach the correct tissue.

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

We need to use animals in which the sensory systems have some strong resemblance to those present in humans since we want to be able to translate our finding in the future to people. The long- term aim is to devise therapeutic intervention to ameliorate or prevent sensory disorders. There are not alternative animals that can be used for this project that have less sentient, while retaining the same genetic tractability and similarities with the mammalian system. However, as part of the work proposed in my additional PPL, we are currently using the less sentient zebrafish to address more general/mechanistical questions about sensory processing. Although a large number of the procedures will be performed following schedule 1 or non-recovery anaesthesia, some are not possible because we require to further use the animals to test the results following the procedure.

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

During our procedures we will carefully monitor the condition of the animals and will apply early endpoints should any notable pain, suffering or distress become apparent. In addition, we are always constantly monitoring our experimental endpoints to determine if the questions posed can be answered through reducing experimental time an individual fish is being monitored, or looking at earlier timepoints in the progression of auditory or vestibular dysfunction.

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

To the best of my knowledge there are no published best practice guidelines for the animal work in this project license. However, we use philosophies of experimental design advocated by the likes of Festing and Wurbel in order to refine our experiments. We also use LASA Aseptic surgery (https://www.lasa.co.uk/wp-content/uploads/2017/04/Aseptic-surgery-final.pdf).

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

I will keep abreast of advances in the field of 3Rs and new 3Rs initiatives through the NC3Rs website and/or attend future 3Rs workshops related to the use of mammalian species.

17. Regulation of cardiovascular development and ageing

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:

Key words

Cardiovascular, Lymphatic, Development, Aging

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

To understand the cellular and molecular processes that control the formation and function of blood and lymphatic vessels during mammalian development, homeostasis 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?

The cardiovascular and lymphatic systems deliver nutrients and remove waste products from every cell in the body, allowing our brains to process information, our hearts to beat and our muscles to move.

Defects in the function of these systems can lead to a broad range of common diseases, including myocardial infarction, stroke, dementia and lymphedema. Conversely, aberrant vascular growth is associated with numerous pathological conditions, including cancer and arthritis. Thus the identification of novel strategies to modulate vascular development,

homeostasis and ageing is of paramount importance.

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

The main outputs of this work will be knowledge about how molecular processes within blood and lymphatic vessels control their development, differentiation, migration, survival and function. The results of these studies will be published in peer reviewed journals, and the publications will always be open-access and available for all to read for free.

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

The immediate beneficiaries of this work will be other academic researchers studying similar biochemical processes that regulate the formation and ageing of blood and lymphatic vessels. More broadly, in the medium-term, it will benefit scientists studying the molecular processes that go awry in diseases of the cardiovascular and lymphatic systems. Most importantly, in the long-term, our work will provide a blueprint for the rational design of cellular therapies that can modulate vascular function in disease, which could be applied to the treatment of stroke, myocardial infarction and lymphedema.

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

Our findings will be presented at national and international scientific conferences and meeting as posters and talks, and eventually published in peer-reviewed journals, and the publications will always be open-access and thus available to all to read for free. Unsuccessful approaches will be discussed openly in appropriate venues. We have an extensive track record of national and international collaboration, and will continue helping other groups with more limited experience in these areas of research.

Species and numbers of animals expected to be used

- Mice: 12500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 animal of choice for these studies because the development of their cardiovascular and lymphatic systems is closely related to human.

In addition, the cardiovascular and lymphatic systems of mice have been studied more extensively that that of any other mammal, and huge numbers of reagents are available for genetic manipulation and subsequent analyses.

Most of the animals are studied during embryonic stages, but some work on the function and ageing of the cardiovascular and lymphatic systems will be carried out using neonates, juveniles and aged mice.

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

Genetically altered mice will be bred, and embryos harvested to allow post-mortem investigation of blood and lymphatic development following genetic manipulation.

Neonatal, juvenile, and pregnant animals may be given gene inducing/deleting substances such as Tamoxifen by commonly used injection routes (subcutaneous, intravenous and intraperitoneal) or orally via a direct bolus to the stomach by a feeding tube or if we require these substances to be delivered over a longer period in the feed or drinking water.

Animals may also be given substances to ablate or label cells via injection which is not expected cause any harm to the animal.

Juvenile animals may be given substances by injection via a slow release osmotic minipump that will induce vessel growth or vascular dysfunction in the adult life stage. So that we can study these effects into old age we will also allow some animals to age up to 24 months.

Animals will be humanely killed at the end of the experiment and tissue collected for analysis post- mortem.

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

Most animals will suffer no adverse effects.

A small percentage of these mice may suffer some adverse effects as a consequences of gene modification (for example, if we modify a gene which plays an important role in adult vascular growth, it is possible that mice may show significant growth deficiencies).

A minority of animals will be injected with substances to induce genetic modifications or inhibit key molecules. These experiments will allow us to study the development, function and ageing of the cardiovascular and lymphatic systems. Impacts and adverse effects from administration of substances are expected to be rare. However, potential adverse effects could include effects due to alterations of gene expression caused by the substances given or adverse effects in direct response to types of substances given. The administration inhibitors of blood and lymphatic vessel function can lead to adverse effects that include hunched posture, dehydration, weight loss and mild tremors. Treatments will be limited to 14 days to limit chronic effects of any treatment.
Additionally, there are physiological changes associated with ageing in mice beyond 12 months of age. These include reduced organ function, rectal prolapse, skin abnormalities, corneal opacity, dental disease and overgrown incisors, degenerative joint disease, weight loss/gain and seizures.

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

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

50% subthreshold, 25% mild, 25% moderate.

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

- Killed

Replacement

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

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

Our project aim is to understand the precise cellular and molecular mechanisms that support the development and maintenance of highly complex vascular networks in different organs and tissues. In order to tackle this question, we have used models such as zebrafish that possess a less complex cardiovascular network. However, it is clear that key differences exist in the processes we wish to study between zebrafish and mammals. Since ultimately our aim is to gain insights into human development, mice are the in vivo system of choice due to the close similarities between mice and humans during development and the number of genetic tools available in the mouse system.

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

As outlined above, we have used less complex systems such as zebrafish embryos to study development of the cardiovascular system. Informed with the knowledge gained from this project, we aim to make blood and lymphatic vessels in a dish from human stem cells, with a view to incorporating as many of these emerging technologies into our research as possible. To supplement our animal work we have established methods to computationally model endothelial cell specification, gene expression and loss of function.

Why were they not suitable?

Our comparative analyses of vascular development in zebrafish and mouse embryos have identified a number of fundamental differences in the cellular and molecular control of early vascular development. Thus, since our aim is to gain insights into human development, zebrafish are not a suitable model.

Cell culture-based models of vascular development are showing a great deal of promise to study vascular development. However, these models remain imperfect and fail to capture the complexities of vascular cell heterogeneity, which involve complex differentiation from distinct cellular sources.

Although we will take full advantage of cell culture, it remains necessary to use animals to capture the real complexity of 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?

In order to estimate the number of animals we will use, we have analysed historical animal usage per year for each licence holder working on this project over the last four years. The estimated number allows for recruitment of new research group members over the duration of the licence.

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

We have used the NC3Rs Experimental Design Assistant and statistical analyses to determine the number of animals that we need to use in order to achieve statistical significance in our experiments. To make the most efficient use of breeding animals for the generation of experimental animals, we have carefully reviewed the crossing schemes required to generate the desired genotypes for analysis. Our institution also supports sharing of useful genetically modified animals such as Cre lines and genetic reporters with other licensees in order to minimise animal usage at the institutional level.

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.

When new genetically modified mouse models become available, we will perform pilot analyses with a small number of animals to assess the functionality of the line, before generating larger cohorts for more extensive analyses. We will maintain as many of these genetic modifications in the same line without causing harmful phenotypes. This will allow us to minimise the number of breeding animals required to generate experimental animals. We are using computer models to simulate mutations in genes before assessing their function in mice.

Refinement

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

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

We have chosen to use mice for these studies for a number of reasons. They are ideal model organisms to investigate mammalian development as their biology is close enough for the implications of our findings to be relevant to humans. Mouse embryonic development is well described and there are many genetic tools available to investigate the function of individual genes, meaning we can identify and characterise developmental processes with relative ease. As our primary interest is development, we will seek to study phenotypes at early developmental stages where the animal is less sentient in order to

minimise suffering. Our analyses will primarily be performed on dissected tissue collected post mortem.

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

Most of our work will be carried out on embryos prior to two thirds of gestation, a stage at which mice are not considered sentient. Our previous analyses of vascular formation in zebrafish embryos demonstrated that the cellular and molecular processes we study are different between fish and mammals, making mouse a more appropriate model.

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

A collaborative relationship with the staff in our animal facility ensures that animals having undergone procedures are closely monitored. New genetic combinations which may develop unanticipated phenotypes will also be closely monitored. In these cases, animals will be monitored daily or twice- daily depending on the situation. Animals exhibiting unexpected harmful effects will be killed using an approved method, except in rare cases where these animals are essential for an experiment, in which case we will seeks advice from the Home Office Inspector.

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

We follow guidance provided in the Planning Research and Experimental Procedures on Animals: Recommendations for Excellence (PREPARE) guidelines to ensure reproducibility and minimal use of animals. Furthermore, the NC3Rs and Laboratory Animal Science Association (LASA) websites provide detailed up to date information of research best practice. We follow the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines when preparing data for publication to ensure that our findings are clearly presented and accessible to others. We will also consider any new publications in a peer-reviewed journal relevant to our field offers refinements to our protocols.

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

Our establishment regularly communicates best practice and new advances in animal handling through newsletters, symposia and annual refresher sessions for project and personal license holders. We have access to a dedicated regional NC3R's manager, in addition to our Named Information Officer. In addition, we will regularly monitor the NC3Rs website for updates (https://www.nc3rs.org.uk/3rs- resources). Finally, we will keep abreast of new advances in advanced stem cell derived cell culture experiments through conferences and publications.

18. Biocompatibility and pharmacology of novel nanotechnologies

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Nanomaterials, Nanotechnology, Biocompatability, Neural Interface, Nanomedicine

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?

Nanomaterials are very small materials with at least one dimension (eg. width, length, height) that is less than 100 nanometres (one billionth of a metre) in size. The overall goal of this project is to design and test novel nanomaterials or nanomaterial enabled devices. We aim to design and identify the nanotechnologies that have the biocompatibility (compatibility with living tissue or a living system by not being toxic, injurious, or physiologically reactive) and functionality to be able to translate into clinical use (therapeutic, diagnostic and monitoring) for a wide range of 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?

The use of novel technologies based on nanomaterials (nanotechnologies) seeks to design smarter solutions with potential to overcome key clinical problems. These could have applications in various diseases including cancer or brain disorders.

However, in order to design nanotechnologies for these applications, it is essential to first understand how these nanomaterials interact within living organisms. We aim to test the safety/biocompatibility and biological interactions of nanomaterials or devices incorporating nanomaterials, first in cells grown outside the animal, and then in healthy animals. In addition to biocompatibility, by studying where the nanomaterials go in the body and how the body reacts, we can learn about their safety or identify potential medical uses, which will inform other researchers interested in using the same tools. It will also inform health and safety protocols for those who may be exposed to nanomaterials during their jobs (occupational) and be relevant for the wider public who may be exposed to nanomaterials present in the environment.

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

The new information gained from this project will be shared in the form of scientific publications, conference communications and through public engagement activities throughout this project. While this work is in the early preclinical stages in the development of medical nanotechnologies, the information gained will allow us to further design and develop nanomaterial and nanotechnology approaches for various *in vivo* and clinical applications.

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

In the short to medium term, many of the nanomaterials being investigated are being produced and used commercially for medicine or other applications. The biocompatibility profiles of these materials are very important to study to be able to provide safety information both to researchers who will further develop the technologies and members of our society exposed daily to engineered nanomaterials present in the environment (natural, occupational, or at home).

Eventually, in the long term, patients will benefit from any effective new technologies developed for the diagnosis, monitoring or treatment of diseases.

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

Publication and communication of our findings will always be the primary aim of this work. The goal of every experiment conducted under this licence will be to generate valid, high quality and therefore publishable results and we will endeavour to ensure that all findings meeting these criteria will be published (majority in open access journals) to inform the wider scientific community.

Species and numbers of animals expected to be used

- Mice: 2500
- Rats: 700

Predicted harms

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

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

Mice are chosen for many of these studies for a variety of reasons, primarily as they are the least sentient mammalian species that will provide data applicable to humans. The similarities between mice and humans in terms of physiology, genetics, immunology and other systems allow us to make reliable predictions about the likely interaction of nanomaterials and nanomaterial devices in humans. Rats are used as an alternative rodent model for some of this work primarily where the larger size provides more anatomically relevant information for nanomaterial device development and biocompatibility testing.

The majority of research will use adult animals as these will have the most closely related biology to adult humans, where these nanomaterials are intended to be used.

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

For the initial testing of nanomaterials, healthy animals without an existing disease or clinical characteristic will be administered (by injection) or implanted (by a surgical procedure) with these nanomaterials via an appropriate route (usually a single administration at the start of the experiment but occasionally repeat administrations throughout). These routes will vary depending on how the nanomaterials are intended to be used as medical technologies, or the likely route of exposure when considering environmental nanomaterials. In the case of nanomaterial interactions with the brain (either as materials or as devices) this initial administration will require a surgical procedure.

Following dosing or device implantation, animals will be monitored by a range of approaches. Outcome measures in the majority of these studies would include one or more of the following: live imaging, blood sampling either to recover nanoparticles, or for assessment of systemic markers, urine or faeces collection and post-mortem tissue histology to assess local and systemic inflammatory reactions, as well as distribution patterns. For many animals, the only procedure performed would be administration of materials, with the assessments done after humanely killing, or from samples collected under terminal anaesthesia.

For nanotechnology based devices, once initial biocompatibility of the devices is carried out (as described above) and we are confident that they are not having any effects beyond standard devices already in clinical use, we can begin recording and/or stimulating from the devices to assess how this may impact the biocompatibility or functionality of the device. These assessments will utilise a number of techniques to assess the state of both the tissue and the animal as a whole. This will include blood sampling to check systemic effects, and behavioural tests. This will allow baseline behaviour of the animal before implantation of the device, assessment after implantation with and without recording for a full comparison. These behavioural tests will be minor and non-stressful for the animal, utilising widely used techniques such as rotarod for motor function. The effects of any neuroprotective or other agents embedded in a material surrounding the electrodes can also be assessed to determine whether their presence has beneficial effects on the tissue. At the end of the experiment, animals will be humanly killed and tissues collected (particularly those in contact with the device) for detailed assessment.

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

From our previous work, we don’t expect the nanomaterials or devices we use to have any

particularly strong adverse effects. Usually the nanomaterials would not be associated with any clinical signs and the effects of these on cells or tissues are only observable when conducting detailed analysis post- mortem (microscopically/histologically/molecularly). Where unexpected substantial reactions occur, animals will be humanely killed as these effects would likely interfere with the aims of the studies. The procedures used to administer the nanomaterials are usually the least invasive possible. Where surgical administration is needed, this will be done under anaesthesia and analgesia/additional support is provided to minimise the effects associated with this.

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

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

- Mice: 50% mild, 50% moderate
- Rats: 100% moderate

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

- Killed

Replacement

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

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

Before materials or devices are tested in animals, they will first be tested in cell cultures, or in relevant *in vitro* systems to check the safety and to help determine the doses that would be safe, or provide a particular effect *in vivo*. Many responses to nanomaterials, including the pharmacology and biodistribution, are driven by complex interactions with multiple cell types as part of whole systems (eg. the immune system, the cardiovascular system etc). These cannot be effectively modelled and integrated in an *in vitro* setting and therefore require the use of live animals.

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

In vitro cell cultures including 3D models and co-culture systems (up to and including organoids which are 3D *in vitro* models that contain multiple cell types and mimic organs more closely).

Why were they not suitable?

Non-animal alternatives such as those listed above can provide important information and are always used in the first instance for all new technologies and nanomaterials. This includes testing in organoid systems which is an ongoing effort by our group. However, none of these systems (including organoids) can effectively recapitulate the complex multi-system interactions of a whole organism, as is required for 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?

Numbers of animals have been estimated in consultation with statisticians using historical data from our own experiments with the same models and approaches with similar nanomaterials or nanomaterial devices.

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

We aim to use the minimum numbers of animals required to adequately and robustly address the research question. This has been determined with support from statisticians and use of rigorous experimental design considerations (as guided by the NC3Rs Experimental Design Assistant). Use of adequate numbers of animals will reduce variability, improve experimental consistency and confidence in outcomes. All assumptions on which sample size estimates are based will be re-evaluated once additional or new data is available from these studies and if necessary numbers of animals required will be revised for subsequent studies.

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

The main way we will reduce the number of animals we use will be to use longitudinal monitoring and live imaging techniques. This will allow us to obtain data from the same animal over time instead of the more traditional method of killing a different animal at every timepoint. Where nanomaterials or nanomaterial devices are being tested for the first time in animals, pilot studies will be run with smaller numbers to ensure safety and provide an initial assessment of effect or functionality that will be used to statistically determine the correct number of animals to use for further investigations. Where possible (eg. device implantation to a hemisphere of the brain) we will use animals as their own control (eg. non-implanted hemisphere). Finally, at the end of each experiment we will collect as many tissues as possible in order to maximise the potential output from 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.

For initial assessment of nanomaterials, or nanomaterial devices, healthy animals without any disease or clinical phenotype will be used. This will minimise the suffering and harm to animals while giving the most clear picture of the effect of the nanomaterial, or nanomaterial device without any confounding factors.

For administration of nanomaterials, the least invasive route that is relevant for the particular application will always be used. Where more invasive routes are necessary (eg surgical administration) this will be scientifically justified and through proper aseptic technique, pain management and careful monitoring is not expected to cause any additional distress or prolonged suffering.

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

The animals proposed are the least sentient mammalian species. The use of non-mammalian species (eg. Xenopus, Danio) would not be appropriate for the clinical translation of the nanotechnologies under development. Mice and rats are the most appropriate for the work being carried out as they have circulatory, nervous and excretory systems very similar to humans, which allows us to model where the materials go, how the body breaks them down and how they are removed from the body in a system similar to humans.

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

Animals undergoing surgical procedures will receive appropriate analgesia to prevent any post- operative pain, will be carefully maintained at a suitable depth of anaesthesia and may also receive additional fluid support to prevent dehydration associated with longer procedures. These animals will be provided with additional husbandry such as mash/wet food, heated housing and careful monitoring in the immediate hours following surgery until normal activity is resumed.

Animals will be group housed and where animals have been individually housed for a particular purpose (post-surgical recovery) these will be grouped as soon as is appropriate.

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

All experiments will be planned and executed with reference to the PREPARE and ARRIVE 2.0 guidelines to ensure effective experimental planning and proper reporting of experiments respectively. We will follow guidance from BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinements and LASA guiding principles for Administration of Substances and Aseptic surgery.

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

The researchers working under this licence will be regularly encouraged to actively stay informed on advances in the 3Rs as is required by the conditions of their PIL. We will regularly check information on NC3Rs website and newsletters and we will attend institutional and regional 3Rs symposia. Any relevant advances, for example refinement of

techniques or approaches, will be readily implemented into this project.

19. Cell signalling in the exocrine pancreas

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

Acute pancreatitis, Chronic pancreatitis, Exocrine pancreas, Calcium signalling, New therapy

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?

The aim of this project is to investigate the mechanisms of acute and chronic pancreatitis in different cell types of the exocrine pancreas to identify potential new therapeutic targets and to test the effectiveness of novel protective substances using *in vitro* and *in vivo* experiments. The obtained knowledge will be essential for future translational studies using 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?

Acute pancreatitis is an inflammatory human disease with considerable risk of death among patients with no specific cure available in clinics. Acute pancreatitis can often progress to the chronic form and pancreatic cancer, which is the fourth most common

cause of cancer related death. Therefore, it is very important to identify mechanisms and therapeutic targets to establish a new treatment for the disease.

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

This project opens new opportunities for the development of preventive and therapeutic measures for both alcohol- and asparaginase-induced acute pancreatitis. Our studies suggest that both the regulation of calcium signalling, and provision of energy supplements can protect against the disease. Testing our hypothesis using in vivo experimental mouse models of acute and chronic pancreatitis are extremely important for developing new drug prototypes and relevant therapies. Using novel calcium channel inhibitors we are going to develop potential anti-pancreatitis drugs. Additional molecular targets for future treatment will be designed. We will test novel inhibitors using in vivo experimental models of acute pancreatitis in mice to establish the most effective dose and regime for the treatment of the disease.

Our recent study provided a detailed mechanism of L-Asparaginase-induced acute pancreatitis and suggested several ways of potential protection. Our work will likely result in improvements of childhood leukaemia treatments.

Results of our work will develop new treatment protocols for acute and chronic pancreatitis as well as for L-Asparaginase-induced acute pancreatitis in future clinical trials.

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

Our work will provide an important step in translation of the results into human clinical trials. Therefore, in the long term, it is hoped that patients suffering from acute pancreatitis will benefit from new treatments with novel drugs.

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

We have well established collaboration with research scientists and pharmaceutical companies worldwide. We will publish our research in high impact scientific journals in compliance with the ARRIVE guidelines.

Species and numbers of animals expected to be used

- Mice: 6 500
- Rats: 180

Predicted harms

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

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

Though most of our research is done initially *in vitro*, the complete understanding of the disease mechanisms under between physiological and pathological conditions is impossible without experiments where pancreatitis can be reproduced using adequate *in vivo* animal models.

Mice and rats remain the most studied animal model in pancreas research that are typically extrapolated to humans due to genetic similarities between rodents and humans. In the non-invasive (non-surgical) acute pancreatitis and chronic pancreatitis rodent models, alcohol metabolites injections and repetitive caerulein injections are amongst the most widely used. These models also demonstrate very high reliability and reproducibility. Other compounds such as substances for potential therapeutic treatments can also easily be added to the design. Transgenic/knockout animals with potential protection against acute and chronic pancreatitis or with expressed fluorescent indicators for measurements of different cellular pathways in health and disease are typically used in modern research to study mechanisms and develop treatments for pancreatitis.

Most experiments will be conducted in wild type (WT) mice (from 4 weeks to up to 3 months, both male and female), as this is the species in which the bulk of research in the field of physiological and pathological calcium signalling has been performed and mouse models of pancreatitis have been developed, with some confirmatory studies in the rat.

Transgenic animals will be used in our work to achieve the aims. It is difficult to overstate the importance of the use of knockouts for the described experiments since their use is the most effective method of testing our hypotheses.

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

In our project we are going to use three protocols.

Protocol 1 “Breeding and maintenance of genetically modified animals” by conventional breeding methods has a mild severity.

Animals produced under this protocol are not expected to exhibit any harmful phenotype. Animals will be bred on site via normal husbandry methods. Animals will then undergo a series of related procedures such as the alteration of their normal diet (to low or high fat), administration of substances and blood sampling.

Transgenic animals that are going to be bred in this protocol are not expected to show higher levels of discomfort during procedures. They are not expected to develop symptoms of acute or chronic pancreatitis due to their genetic status.

Animals from this protocol will be used in Protocol 3 for induction of acute and chronic pancreatitis with moderate severity.

Protocol 2 “Breeding and maintenance of genetically modified animals” by conventional breeding methods has a moderate severity.

Animals will be bred on site via normal husbandry methods. Some genetically altered animals may show some effects such as being slightly smaller. Animals will then undergo a series of related procedures such as the alteration of their normal diet (to low or high fat), administration of substances and blood sampling.

Transgenic animals that are going to be bred in this protocol are not expected to show higher levels of discomfort during steps under Protocol 2 except Bcl-2 KO mice. Due to their genetic status, Bcl-2 KO may be embryonic lethal or lethal before adulthood (up to 3-4 weeks), and such lines will be maintained as heterozygotes. They are not expected to develop symptoms of acute or chronic pancreatitis due to their genetic status.

Animals from this protocol will be used in Protocol 3 for induction of acute and chronic (except Bcl-2 KO) pancreatitis with moderate severity.

Protocol 3 “Pancreatitis induction and possible treatment using Isolated, perfused and exteriorised, vascularised pancreas, diet/drink protocols” have a moderate severity. Wild type animals and mice from Protocol 1 and 2 will be used.

What will be done to animals: food withdrawal, low or high fat diet

the administration of substances subcutaneously or by intraperitoneal or intravenous injections

induction and maintenance of terminal general anaesthesia with no recovery: experiments will be performed while the animal is anaesthetised

withdrawal of body fluids will be undertaken using a combination of volumes, routes, and frequencies that of themselves will result in no more than transient discomfort and no lasting harm

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

Protocol 1 “Breeding and maintenance of genetically modified animals”

Transgenic animals that are going to be bred in this protocol are not expected to show higher levels of discomfort during procedures. They are not expected to develop symptoms of acute or chronic pancreatitis due to their genetic status.

Protocol 2 “Breeding and maintenance of genetically modified animals”

Transgenic animals that are going to be bred in this protocol are not expected to show higher levels of discomfort during steps under Protocol 2 except Bcl-2 KO mice. Due to their genetic status, Bcl-2 KO may be embryonic lethal or lethal before adulthood (up to 3-4 weeks), and such lines will be maintained as heterozygotes. Under a moderate Protocol 2 we need to breed genetically altered strains such as Bcl-2 KO that may develop some clinical signs described above due to their genetic status in order to investigate the protective effect of Bcl-2 family proteins in development of acute pancreatitis (Protocol 3, moderate). They are not expected to spontaneously develop symptoms of acute or chronic pancreatitis due to their genetic status.

Protocol 3 “Pancreatitis induction and possible treatment using Isolated, perfused and exteriorised, vascularised pancreas, diet/drink protocols”

Adverse effects for the animals during acute and chronic pancreatitis induction: Adverse effects for the animals during acute and chronic pancreatitis induction:
Some animals that are models of acute pancreatitis may experience unbalanced movement or pain, but this will be short lived. Animals will be monitored and given supplements and pain relief. Some transgenic strains could potentially show (less than 5%) higher levels of discomfort during Protocol 3 steps due to their genetic status (such as Bcl-2 KO mice), although they are not expected to spontaneously develop acute or chronic pancreatitis. Therefore Bcl-2 KO animals will be not used for induction of chronic pancreatitis (Protocol 3).

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

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

Protocol 1 – mild severity - less than 1%, remainder will experience sub-threshold

Protocol 2 – moderate severity - up to 30 %, remainder will experience mild

Protocol 3 – moderate severity:

deleterious effects and the likely incidence of pain etc. - less than 5% lack of grooming, or change the natural and provoked behaviour - less than 5%

altered movement due to administration of acute pancreatitis inducing substances - up to 30%

discomfort due to repeated intraperitoneal injections - up to 70 %

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

- Killed

Replacement

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

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

Mice or rats will have to be used to provide the necessary data to achieve our aims.

In some of our experiments we will be required to induce *in vivo* models of pancreatitis for investigation of the mechanisms of disease and to test different protective substances.

The use of freshly isolated pancreatic acinar cells and lobules provides a large amount of *in vitro* work from a relatively small number of animals, and the later work in living animals is carefully designed to minimise the number of animals necessary to achieve the data we need.

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

Experiments using wild type and transgenic mice are also crucial to our studies. Whenever possible alternatives will be used (pancreatic cell lines AR42J, PANC1, etc).

Where possible, we will use human tissue biopsies as a replacement and reduction for animal work for some types of experiments. Previously we have performed experiments to investigate the mechanism of CCK action using isolated pancreatic acinar cells from human tissue biopsies.

Why were they not suitable?

Unfortunately, pancreatic cell lines have a different origin and properties as compared to freshly isolated pancreatic acinar cells and pancreatic tissue.
There is a range of limitations in using the human pancreatic biopsies to study acute and chronic pancreatitis:

First, human tissue taken after pancreatic cancer surgery represents a healthy part of pancreas. It could give a unique opportunity to study physiological processes but cannot be used to study experimental pancreatitis. Only in vivo animal models allow the investigation of the development and progression of the disease.

The second limitation is that human samples must be delivered to the lab very quickly, frequently and on a regular basis. These conditions were very difficult to meet even when our lab in past was physically located near the operation theatre in the pancreatic cancer centre in the University of Liverpool. In fact, only one sample out of nearly one hundred gave meaningful results during 3 years of the project. Samples that have been delivered with a slight delay (>10min) were not usable due to massive cellular injury.

Third, human samples are often contaminated with blood during surgery, leading to pre-activation of pancreatic tissue with active blood components resulting in high levels of cellular necrosis.

Reduction

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

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

We will use minimal sets of animals for the modelling of acute and chronic pancreatitis and few animals for in vivo anesthetised mouse studies.

We are going to share cell preparations between members of our research group to reduce the number of animals to the absolute minimum.

The design of individual experiments will involve a collective approach, which maximises the information obtained from the minimum resource.

The experimental sample size calculation includes:

Effect size – minimum difference you want the experiment to be able to detect (0.7).

Variability – estimate based on previous experiments.

Power – percentage of true positive the experiment can detect (90%).

Significance threshold – probability of obtaining a false positive (p=5%).

For each our experiment, we plan an experimental protocol based on:

main objective(s);

a description of detailed experimental treatments, the sample size of the experiment, and the object; an outline of the analysis of the results.

Such traditional practice allows us to keep animal numbers to the minimum and produce high quality science.

For most of our quantitative experiments, sample sizes will be set using power analysis, i.e., the significance level will be set at 5%, and the power 90% (please see the ERA report below). However, the exact numbers of animals required will vary with the particular experimental design, robustness of effect, etc. For the qualitative experiments, the amount of material required will be the minimum necessary to provide an adequate description of the phenomenon (eg, stellate cells, immune cells and pancreatic neurons activation).

Justification of animal numbers for Protocols 1 and 2:

Resources that we are using for calculation of breeding numbers:

Breeding colony size planning worksheet from Jax labs

www.jax.org/jax-mice-and-services/customer-support/technical-support/breeding-and-husbandry- support/colony-planning
NC3Rs Breeding and Colony

www.nc3rs.org.uk/breeding-and-colony-management-frequently-asked-questions#faq4

For a typical experiment we require approximately 30-40 KO mice per year for each strain. Therefore, for example, in our breeding and maintenance Protocol 2 with moderate severity (up to 3 strains in total) we will use up to 200 transgenic animals per strain with average neonate mortality rate 18% (Figure 1).

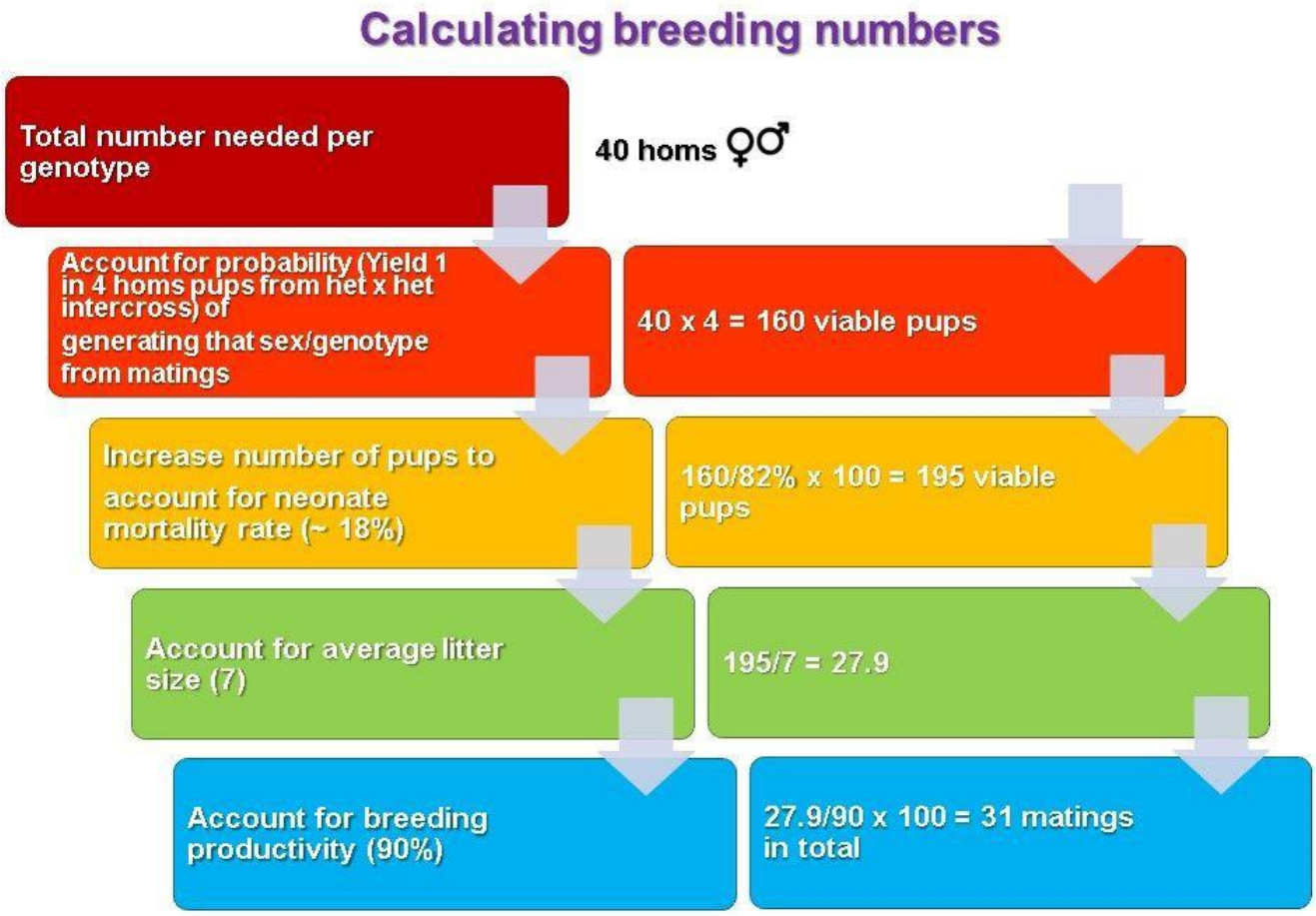


Figure 1. Example of calculation of breeding numbers for transgenic animals with probability of 1 in 4 homozygot pups from het x het intercross and average neonatal mortality rate 18% (Protocol 2, Bcl-2 KO strain, moderate severity)

Justification of animal numbers for Protocol 3:

Numbers of animals have been calculated according to the sample size and variability based on our experimental experience and published data as well as recently run optimisations.

The sample size is determined using the sample size calculator for unpaired t-test provided in EDA. Therefore, a typical experimental design for induction of acute or chronic pancreatitis in mice (Protocol 3) to accommodate two groups of animals: negative control group (no pancreatitis induction) and experimental disease model. As calculated by the EDA sample size calculator the experiments will require 12 mice/rats per group. In a separate series of experiments we will be using up to 3 models for acute pancreatitis and up to 2 models for chronic pancreatitis (up to 6,500 mice/180 rats).

Effect size with small effect – 0.5

Justification for effect size - power calculation to detect an effect size of 0.5 or greater, variability (SD) 0.35 with 90% power, at a 0.05 significance level.

Example of experimental design is provided in EDA report (available on request).

What steps did you take during the experimental design phase to reduce the number

of animals being used in this project?

We will be using our own experience with the help from a free online tool from the NC3Rs The Experimental Design Assistant (EDA), which will guide us through the design of our experiments to ensure that we use the minimum number of animals consistent with the scientific objectives, methods to reduce subjective bias, and appropriate statistical analysis. Feedback and advice on experimental plan will be used to optimise the procedures. We will also discuss our plans with NVS and NACWO and address their feedback in designing and use of animals for our study.

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

We will use pilot studies to establish the most effective protocols and concentrations of substances.

Refinement

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

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

It is well known that late stages of pancreatitis are accompanied by pain, but that the early stages are often asymptomatic, according to human studies. Therefore, when *in vivo* AP models will be used, this will be done only in the early stages of AP when they should not experience significant pain or enduring discomfort. Protocols, therefore, can be limited to mild or moderate severity with appropriate analgesia being used wherever necessary and possible. The *in vivo* protocols will be optimised to refine the number of animals, doses and time of disease development to get statistically significant results for our study. Only a very small number of confirmatory experiments will have to be conducted in rats to compare with results obtained on mice. In many recent publications, research has been performed using both mice and rats to deliberately induce experimental acute pancreatitis. Therefore, there is a high probability that experiments using rats will be required by the reviewers of our grants and papers to confirm results obtained using mice. In a very small number of our experiments, we are going to develop pancreatitis in genetically altered animals, although the stimuli required for inducing this condition are similar to those in wild type mice (e.g. intraperitoneal caerulein administration). The dosage and timing of any stimulus will be refined to minimise suffering and to ensure that the earliest events are captured. In knockout mice, we will initially restrict experimental intervention to terminal procedures under surgical anaesthesia, and this will apply to the majority of experiments where most of the physiological and pathological data can be obtained with only minimal suffering. Again, some animals will receive pre-treatments prior to experimental intervention with exteriorisation of the pancreas under terminal anaesthesia.

In the vast majority of our experiments, animals will need to be maintained for up to 10 weeks (chronic pancreatitis models). We will optimise the protocols using pilot studies to

adjust our approaches and to minimise the time of pancreatitis development.
There is a possibility to employ available transgenic animals with spontaneously developing chronic pancreatitis. Three genetic models of chronic pancreatitis do exist, namely Interleukin 1b transgenic mice (elastase sshIL-1b mice), SPINK3-deficient mice and PERK-deficient mice, providing an alternative to repeated intraperitoneal injections. Unfortunately, the first two of these are not available commercially, in the third, severe CP is spontaneously developed. PERK-deficient mice are also associated with many other systemic health problems affecting the investigation of the upstream mechanisms at the early stages of the disease, so this would not be an acceptable model to use. An analgesic regime will be applied in agreement with the NVS in experiments with acute and chronic pancreatitis induced in animals by intraperitoneal administration of specific drug(s) due to the risk of animals experiencing pain.

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

Mice are the chosen species for most of the work because a) appropriate knockout and mutated models are available in mice, b) standard models of induced pancreatitis have been successfully developed in mice, c) importantly, pancreatic cells and tissue from mice show a great similarity to isolated human pancreatic cells and fragments of human pancreas.

In many recent publications, research has been performed using both mice and rats to deliberately induce experimental acute pancreatitis. Therefore, there is a high probability that experiments using rats will be required by the reviewers of our grants and papers to confirm results obtained using mice.

There are some differences between mice and rats with regards to pancreatitis: alcoholic pancreatitis in rats is very similar to humans as opposed to mouse. Alcohol and dietary fat feeding models in rats (non-invasively) have been established in 1988 and since then were in use to study acute and chronic pancreatitis.
Alcohol diets in mice are not sufficient to induce the disease, therefore so called "alcohol" model must rely on additional chemicals mixed with alcohol (POA/alcohol) and IP injections.

Rat model is needed not just for confirmation but due to differences in alcohol-induced pancreatitis mechanisms in mice and rats. Referees can argue that mouse model is unnatural and ask for a confirmation in a human-like model. We do occasionally receive such requests/criticism for our grants and papers. Mouse models are still very popular; therefore, fortunately, we do not need to confirm every result in a rat model.

Most influential review in the area of pancreatitis emphasises: "Care must be taken to select the model most appropriate for answering each question or testing each hypothesis".

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

To reduce the amount of injections, pilot studies will be applied to determine a chemical cross-reaction and solubility of different substances, such as caerulein, calcium channel blockers and other substances for the possibility of simultaneous intraperitoneal injections. We have looked into the possibility of avoiding the repeated intraperitoneal injection regime by the use of an osmotic mini pump to deliver the caerulein or alcohol metabolite

mixture into the abdominal cavity. However, this method has problems that directly interferes with our investigation: 1) It has been designed for the continuous chronic infusion of the substances for long term. In our case, we need to deliver substances not continuously but in the form of single acute hyperstimulation to mimic the recurrence of acute pancreatitis attacks and lead to development of chronic stage of the disease.

It has been shown previously that osmotic mini pump implants can cause desensitization of the pancreatic secretion, which directly interferes with our research.

There is a risk of spontaneous development of acute pancreatitis as a result of various surgical procedures in humans with unclear/poorly studied mechanisms.

Another problem with mini pump usage in our experiments is the instability of the substances i.e. hormone analogue or alcohol metabolite or asparaginase enzyme that would have to be kept in the pump for many hours.

With the help of Chief Technician we have investigated the possibility of using implantable micro infusion pump, but the version commercially available to us so far is designed for rats only, while maximum flow rate is too slow for our requirements.

When a suitable micro infusion pump will become commercially available, we are going to adopt whenever possible this method for administration of drugs in our models.

In all cases, pain-reducing measures will be sought and implemented wherever possible in collaboration with the animal facilities team to improve the animals' experience throughout their life, e.g. by enriching the cage environment. We will use a substitution of drinking water with alcohol that could potentially reduce amount of alcohol injections. Some intraperitoneal injections of substances will be combined with injections of pancreatitis-inducing agents when possible to reduce number of injections.
Cascade approach for the use of novel substances:

We will use a cascade approach for use of novel substances across all our work:

Where published safety and doses data are available from long term toxicology studies then no additional precautions are required.

Where there are no such data, but previous experience derived from longer term administration studies has provided such information, then no additional precautions needed.

Where no data exist, animals are dosed individually with an appropriate lag between animals using gradually escalating doses.

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

To assist with planning animal research and testing we will follow published guidelines such as the PREPARE guidelines:

http://journals.sagepub.com/doi/full/10.1177/0023677217724823;
https://norecopa.no/prepare
Other resources will be also used such as guidance and publications from the NC3Rs and

Laboratory Animal Science Association.

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

We will keep up to date with the latest news from the NC3Rs (https://www.nc3rs.org.uk/news) and monitor scientific publications, i.e. “The NC3Rs publishes its strategy for the next three years (https://nc3rs.org.uk/who-we-are/our-strategy). We will use a local networks and information sharing with users of the animal facility at our home establishment including NACWOs.

20. Development of haemostatic medical devices

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Haemostasis, Medical device, Trauma, Bleeding, Coagulation

Animal types	Life stages
Rats	adult
Mice	adult

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

To determine the effectiveness (efficacy), toxicity, and biodegradability of a novel medical device to stop blood loss (haemostasis).

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

Why is it important to undertake this work?

Uncontrolled bleeding is a major cause of death worldwide following accidents, battle field injuries and surgery. Many of these deaths occur in young people and are considered preventable with better intervention. Typical treatment involves devices such as bandages which soak up blood, stick to the wound and subsequently tear the dried wound. We are developing modified bandages that reduce blood loss by not soaking up blood, triggering

clotting and not sticking to drying wounds. Our work will lead to better blood loss prevention in a wide range of circumstances leading to fewer deaths.

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

We will generate new data sets that will be published in scientific journals and at scientific conferences that will demonstrate efficacy and safety of the new bandages. We will generate intellectual property and patents for future commercial development. We will develop prototype medical devices for further testing and commercialisation (making into saleable products).

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

Ultimately our work will benefit patients requiring treatment for bleeding. To deliver benefit, our products will need to be commercialised and we have funding to start this process although it will take several years. We will focus initially on delivering benefit in trauma settings, where we aim to use our bandages to stabilse victims' wounds and prevent deaths before reaching hospital. We will then extend benefit to other bleeding scenarios such as surgery. To deliver these benefits, we need to first show that our bandage can produce advantages over existing products in bleeding scenarios. These data will also be of interest to doctors, a range of health care workers and scientists in the fields of biological science, medicine, bioengineering and materials science. If our animal studies are promising, we will progress to clinical trials and will aim to hold discussions with commercial organisations with an interest in producing novel bleeding prevention devices. In addition to human benefits, our work may benefit animals undergoing surgery or treatment for trauma as our bandages could be adapted for veterinary use. Our research will therefore also benefit veterinary surgeons and nurses.

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

We will engage with organisations that promote commercialisation of scientific technology such as the Institute for Deep Tech Entrepreneurship. We will also seek additional funding from a range of charitable funders, research councils and commercial organisations. We will share our research via social media and through outreach events such as Pint of Science. We will also publish our findings in freely accessible scientific journals.

Species and numbers of animals expected to be used

- Mice: 100
- Rats: 250

Predicted harms

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

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

We are using adult mice and rats as we need to work with mammals that replicate human bleeding and the prevention of blood loss through having a system for delivery of blood around the body (circulatory and cardiovascular systems) that is like that in humans. We also need to work with mammals with a large enough blood volume so that blood soaking

into devices is measurable. Adult rats have been chosen as we will be able to measure their blood loss during procedures and so that the cardiovascular system is fully developed and reasonably represents that in adults and children. In addition, we need to work with mammals that will replicate human physiology (organs, tissues etc.) and the immune system in order to mimic the likely human response to left-over materials or fibres from our bandages. For these experiments we will work with mice because their immune system is known to be very similar to human. Adult mice have a mature immune system and provide a large enough skin area to allow injections of substances to be given multiple times.

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

Rats will be put to sleep permanently with anaesthetic and the tail vein cut to cause bleeding. A test bandage will be applied for a few minutes until bleeding stops. The bandage will be removed and then blotting paper will be applied to the wound to capture any remaining blood loss. The process will be repeated up to five times with different bandages. A blood vessel in the thigh (femoral artery) or the liver will then be exposed and cut to cause bleeding. The test bandage will be applied to the bleeding area. The animal will then be killed. Alternatively, mice will be injected under the skin with the microscopic parts of bandages, called nanofibers, which are too small to see with the naked eye, while under temporary general anaesthetic. Up to 5 injections will be given to each animal in rapid succession following shaving of the injection site which will then be marked with non-toxic marker pen. Mice will be monitored for up to 6 weeks and then killed. During the monitoring period the shaved site will be retained with additional shaving and re-application of marker while the animal is awake. Mice will then be killed and tissues will be taken and tested to determine that nanofibers break down in the body (biodegradation) without causing harmful effects (toxicity).

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

Animals in bleeding experiments will experience mild discomfort during the process of being put to sleep with anaesthetic. During biodegradation and toxicity experiments, animals will experience brief discomfort during the process of being put to sleep with anaesthetic and during recovery, and then minor discomfort due to the presence of nanomaterials under the skin, which may cause some irritation.

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

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

For all animals : 50% mild; 50% 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?

We need to mimic bleeding in humans and other mammals, including blood vessel damage and tissue injury, and this is only possible by causing a wound in an intact, living animal. Animals are also needed so that we can study what happens if any material from bandages remains under the skin, what the immune response if, and how these materials biodegrade. There are no non-animal alternatives to these biological processes.

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

We have considered clotting experiments using human blood and plasma and will use these experiments to select compounds for further study but they are not suitable to determine the efficacy of our product in reducing blood loss. We are also measuring biodegradation of substances in human plasma and on agar gel plates to mimic degradation of device products during wound healing.

Why were they not suitable?

Studies with human blood do not mimic whole organism blood pressure or vessel damage and so cannot determine efficacy in an injured and bleeding human. Biodegradation in plasma and on gels will partially mimic degradation of fibers in humans but it will not mimic the internal environment of the body and the immune response is absent.

Reduction

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

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

We have estimated animal numbers based on the anticipated number of experiments, the numbers of experimental and control groups and the numbers of animals in each group. The estimates are based on similar studies reported in the field.

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

We used the NC3Rs experimental design assistant and consulted with the college statistical design service to design experiments with the fewest animals while returning high quality data.

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

Our materials will first be tested in non-animal models involving human blood and plasma clotting as well as degradation of fibers in non-animal models. Only a small number of materials that are effective in these models will be carried through to animal studies. In

addition, more serious injuries are performed after the tail bleed experiments so that the serious bleed is not so great that it immediately kills the animal, this also has a reduction benefit. For biodegradation experiments, multiple injections will be given to each animal to allow comparisons to be made and leading to reduced animal numbers.

Refinement

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

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

We will be creating cuts in the tails of rats to cause blood loss. In some rats, we will also be exposing the liver and femoral artery and injuring them to cause blood loss. All bleeding procedures will be conducted under terminal anaesthesia. We will also be injecting substances under the skin of anaesthetised mice who will recover and be monitored for several weeks. We will work with products that are thought to be non-toxic as they are also likely to be suitable for human use. In these experiments, we are mainly testing for biodegradability but lack of toxicity will also be confirmed. All proposed procedures are carefully adapted from a scientific and 3Rs perspective from successful models reported in the literature.

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

All experiments in rats are conducted under terminal anaesthesia.

Mice will undergo procedures when concious as some procedure will be preformed under recoverable anaesthesia. These experiments will take place over a period of weeks meaning that aneasthesia is not feasible, and we avoid using painful procedures by not using materials that are thought to be toxic.

Eventually, our products will need to be tested in larger animals, but we are not yet at that stage of development and so are not applying to use larger animals within this license.

We are unable to work with non-protected or less sentient animals such as zebrafish due to the need to induce measurable blood loss and apply a bandage in a way that models human treatment. This would be impossible in these species.

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

Bleeding protocols are conducted under general anaestheisa and we will work with the Named Veterinary Surgeon to identify and use the most refined anaesthetic regime for the procedure in order to ensure effective anaesthesia.

Animals receiving injections will be monitored daily by the PIL holder. Where problems arise, we will consult the NACWO and veterinary surgeon. Animals will be humanely killed

if found to be distressed or in pain as this is not expected. When shaving animals, we will do so on one side of the body only to minimise distress and hair loss and we acclimatise animals to handling and use positive reinforcement (edible treats) to make the overall experience more positive for the animals. Although we have stated that the maximum number of injections per animal will be 5, we will strive for an experimental design involving fewer injections by conducting extensive non-animal studies to rule out some materials prior to animal studies.

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

LASA guidelines PREPARE guidelines and ARRIVE guidelines.

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

I regularly attend conferences and events organised by NC3Rs and will continue to do so and will continue to read NC3Rs newsletters as well as internal newsletters from College 3Rs committee and our animal facility. I will regularly engage with in-house 3Rs training available to all staff within my institution.

21. Host-pathogen interactions in bovine digital dermatitis

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
  - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes

Key words

Cattle, Lameness, Pathogen, Genetics, Digital dermatitis

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?

Digital Dermatitis (DD), a major cause of cattle lameness, is a painful, infectious, foot skin disease that compromises the welfare of hundreds of millions of farmed ruminants. We aim to improve our understanding of host-pathogen interactions and DD pathogenesis, in order to advance breeding programmes and identify novel routes for future targeted vaccine and drug development.

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

Why is it important to undertake this work?

Mounting welfare and cost issues brought about by increasing prevalence of DD in bovine populations both nationally and worldwide demonstrate the timeliness of the proposed project. State of the art “omics”, data analyses, and novel in vitro disease models will be

used to achieve a mechanistic understanding of host-pathogen interactions and DD pathogenesis, in order to advance breeding programmes and identify novel routes for future targeted vaccine and drug development. This will improve animal health and welfare, potentially reducing further development of antimicrobial resistance, and support the production of healthy food. Associated decrease in involuntary culling of animals and increase in cattle longevity will indirectly contribute to reduction of greenhouse gas emissions and improve the sustainability of the sector. Outcomes will also contribute towards the improvement of the bovine reference genome annotation, advancing future genomic studies in cattle.

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

We will provide novel insights into the genomic architecture of DD.

We will identify cell populations with a key role in disease resistance and progression, candidate genes with significant effects on host resistance to DD, regions with regulatory functions and biological pathways encompassing these genes.

We will survey differences in immune responses and use novel in vitro models of the disease to identify differences in host cellular responses, between animals of different genetic merit for resistance to DD to underpin a mechanistic understanding of host resistance which should inform the development of novel preventive and therapeutic approaches.

We will provide a list of prioritised candidate genes and genetic variants and will validate some of the genes and genetic variants with a potential causative role in DD resistance and progression providing further insights on the genetic architecture, the pathogenesis and the pathobiology of the disease.

We will assess the expected rate of change in DD incidence under different selection scenarios and identification of the best strategy for improvement of foot health simultaneously with cow productivity, fertility, overall health, and longevity.

New knowledge, results and methodologies will be widely disseminated in the form of (i) peer-review articles in international journals, (ii) presentations in scientific meetings and conferences both nationally and internationally, and (iii) participation of the researchers in workshops, networks and relevant academic forums for information exchange.

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

State of the art “omics”, data analyses, and novel in vitro disease models will be used to achieve a mechanistic understanding of host-pathogen interactions and DD pathogenesis, in order to advance breeding programmes and identify novel routes for future targeted vaccine and drug development. This will improve animal health and welfare, potentially reducing further development of antimicrobial resistance, and support the production of healthy food. Associated decrease in involuntary culling of animals and increase in cattle longevity will indirectly contribute to reduction of greenhouse gas emissions and improve the sustainability of the sector.

New fundamental knowledge and insights will directly benefit the applicant organisations as well as the scientific community as a whole, especially since generated data and developed methodologies and in- vitro models will become publicly available.

Generated data and results will be shared with the FAANG, BovReg and farmGTex consortia (see relevant LoS) to enhance cattle genome annotation and therefore advance future cattle genetic studies, leading to considerable improvements in cattle health and welfare.

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

New knowledge, results and methodologies will be widely disseminated in the form of (i) peer-review articles in international journals, (ii) presentations in scientific meetings and conferences both nationally and internationally, and (iii) participation of the researchers in workshops, networks and relevant academic forums for information exchange. The interdisciplinary nature of the project suggests that these publications and activities can reach and potentially benefit a wide range of specialist teams worldwide in the above-mentioned scientific fields. Furthermore, results, in vitro models, and sequencing data will readily become publicly available for future use by the scientific community.

Species and numbers of animals expected to be used

- Cattle: 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.

Infectious foot health issues emanating from digital dermatitis (DD) are a major animal welfare concern and a primary source of economic losses to the dairy cattle industry. We can only address our objective of in-depth characterisation of the genomic architecture of host DD phenotypes using the target species. The predominant dairy breed in the UK and worldwide, Holstein, has been selected for this study.

Our ongoing research has shown that 45% of examined young animals (10-12 months old) were either already infected or became infected within three months from enrolment; approximately half of the infected animals subsequently became chronically infected. These findings highlight the need to include young animals in DD research as first infection events can occur very early in life.

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

We will use 500 heifers from one or two dairy cattle farms. The heifers will be safely handled in specialised handling crushes at 6 time points and for a six month period:

At enrolment (heifers will be enrolled approximately 10 months after their birth)

Monthly for five months post enrolment

At each time point the following regulated procedures are to be performed:

Blood sampling while animals are restrained in appropriate handling facilities. Blood

sampling of dairy heifers is associated with mild discomfort which is minimised when performed by experienced operators.

Foot biopsy (only on a subset of 72 animals). The procedure to take place on farm of origin and using appropriate handling facilities local regional anaesthesia will be administered in order to desensitise the foot. A single, 1cm diameter punch biopsy will be taken from the foot skin at the typical DD infection site (plantar aspect of the foot and between heel bulbs) as previously described in the literature. Systemic analgesic agents appropriate to the species will be administered. Pain and discomfort from the procedure are likely to be minimal and will not cause lasting harm. Biopsy wound could become infected but this will be monitored/ managed by our research team.

We will obtain biopsies from 24 animals genetically resistant to DD (animals will be genotyped and genotypes will allow calculation of the DD genetic index, a genetic evaluation of animals' resistance to DD) that will remain healthy throughout the study. Furthermore, 48 genetically susceptible animals will be biopsied at the first DD infection event. Our preliminary data suggest that approximately half of these animals will recover from the infection while half will become chronically infected; these animals will be sampled again two months after the initial biopsy to study further the healing process and compare chronic cases with recovered ones. A subset of 15 of these animals (5 per group/ randomly selected) will also be biopsied one more time to provide material for skin cell isolation.

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

Blood sampling: There are unlikely to be any adverse effects of note associated with blood sampling. There will inevitably be the transient mild discomfort associated with venepuncture and the small possibility of haematoma formation.

Foot skin biopsy: Pain and discomfort from the procedure are likely to be minimal. Biopsy wound could become infected.

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

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

Mild

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

- 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?

Infectious foot health issues emanating from digital dermatitis (DD) are a major animal welfare concern and a primary source of economic losses to the dairy cattle industry. We can only address our objective of in-depth characterisation of the genomic architecture of host DD phenotypes using the target species. The predominant dairy breed in the UK and worldwide, Holstein, has been selected for this study.

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

There are currently no non animal alternatives that would allow us to achieve our objectives. The created cell lines will eventually allow us to study host pathogen interactions in vitro therefore reducing the use of animals.

Why were they not suitable?

There are currently no non animal alternatives that would allow us 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?

A large amount of already available data will be used. A prospective sample of 500 heifers will be added. This number was calculated based on our existing data and knowledge of the disease incidence on the collaborating farms.

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

We are going to utilise already existing datasets and will therefore significantly reduce the number of animals enrolled in this project.

The study has been designed by experts in quantitative genetics and training in Epidemiology

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

Use of pre-existing datasets. Use of in vitro models of the disease.

Refinement

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

of the project.

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

Cattle are currently the major species affected by the diseases under investigation in this project.

Only minor adverse effects may be associated with blood sampling. There may be some transient minor discomfort. Some cows may develop a small haematoma at the sampling site; in this case an alternative site will be selected for subsequent sampling.

Foot skin biopsies will be taken at the typical DD infection site (plantar aspect of the foot and between heel bulbs) as previously described in the literature. Skin biopsies are necessary for our transcriptomics studies and will also be used for the development of cell lines that will then allow in vitro studies of host pathogen interactions.

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

We can only address our objective of in-depth characterisation of the genomic architecture of host DD phenotypes using the target species.

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

Only minor adverse effects may be associated with blood sampling. There may be some transient minor discomfort. Some cows may develop a small haematoma at the sampling site; in this case an alternative site will be selected for subsequent sampling. Animals will be handled and restrained for the procedure by experienced veterinary surgeons. Personal licensees carrying out the procedure will be experienced veterinary surgeons. Severity of the procedure is mild.

Foot skin biopsies will be taken at the typical DD infection site (plantar aspect of the foot and between heel bulbs) as previously described in the literature. The procedures will be performed by trained veterinary surgeons and with the appropriate use of anaesthesia and analgesia. The severity level of the regulated procedure is classified as mild and no long-term welfare issues are expected to arise from the study. Animals will be closely monitored by farm staff and the research team and our previous experience suggest that the biopsy site will be healed within days from sampling. The biopsy site will be bandaged for the first two days post biopsy. There is a small chance of infection; infected wounds will be treated appropriately by the research team (at least one licensed veterinary surgeon will be part of the research team).

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

We will consult the Prepare checklist. Reporting will follow the Strobe statement checklist (reporting for observational studies).

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 newsletter and will keep up with advances in the 3Rs. If we do come across anything that could be implemented in our project we will consider it.

22. Infection model validation and 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

Infection, Antimicrobial, Antibacterial, Antiviral, Antifungal

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim of this project is to introduce, optimise and refine infection models for use in the Establishment’s project license to support the development of antibacterial, antiviral and antifungal treatments and vaccines on behalf of pharmaceutical and biotechnology companies and academic establishments.

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

Why is it important to undertake this work?

Although there have been tremendous advances in the treatment of infectious diseases

there are still many developing and unmet clinical needs.

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

In addition to antibiotics, there is a growing resistance problem with resistance to antifungal drugs, causing a significant rise in the number of deaths, particularly in clinically vulnerable groups such as pre-term babies, HIV infected and other immunosuppressed patients. It is estimated that over 300 million people of all ages suffer from serious fungal infection each year resulting in above 1.7 million deaths globally.

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

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

Individual species of infectious agents can have highly varied sub-strains, which demonstrate very different anti-infective resistance and growth characteristics within the animal model which have to be identified separately. The work conducted under this project licence is critical to supporting the animal models used in the early stages of developing new treatments to these three global public health threats.

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

The studies performed under this licence will support the work carried out on the Establishment's efficacy license and therefore contribute to the early stages of antimicrobial drug development projects. This would not be possible without the availability of optimised infection models that mimic or are surrogates of the human disease and are able to inform on the choice of suitable candidates for clinical trials.

This Infection Model Validation and Development licence will enable the Establishment to validate or refine models that are currently available for use in our anti-microbial efficacy project licence and, when necessary, to introduce new models (bacterial, fungal, viral and parasitic microbes) to ensure the broadest service is available to the drug discovery industry to meet current unmet needs with the best possible balance of cost/benefit. Where significant results are not subject to confidentiality agreements they will be communicated more widely at scientific meetings or published in peer reviewed journals.

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

In the short term clients, project teams, and funding bodies will be able to access a wider range of high quality models to enable progression decisions to be made, to better target their limited financial resources on to the potential treatments with the highest likelihood of clinical success.

This will feed into the use of the anti-microbial efficacy licence to allow the termination of projects with little chance of success (either due to lack of relevance of the target or the inability to develop a potential medicine that has a clear beneficial effect in animal models) will also mean that animals will not be used in safety assessment and human volunteers will not be put at risk in clinical trials.

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

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

Newly introduced and validated models will be shared with clients to enable them to improve the quality of their research programmes, and potentially broaden the scope of application of their targets.

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

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

Species and numbers of animals expected to be used

- Mice: 6300
- Rats: 400
- Guinea pigs: 100
- Hamsters (Syrian) (Mesocricetus auratus): 50
- Other rodents: No answer provided

Predicted harms

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

These animal studies serve as a bridge between computer or cell-based experiments, and efficacy testing of novel therapeutics in animals prior to human clinical trials.

In the majority of cases adult animals will be used as these are representative of the majority of the patient population. Rarely, where a specific infection or treatment focusses on the paediatric population, juvenile or neonatal animals may be used to optimise models for efficacy testing of novel therapeutics under a separate project licence.

Most animals used in this project will be mice (occasionally including genetically altered

animals) as these have been demonstrated to provide information that enables decisions to be made on the progression of treatments. Their reactions to infections and response to antimicrobial treatments have shown good alignment with those seen in human patients. Using genetically altered animals allows the investigation of human-specific targets and treatments for these targets.

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

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

For model optimisation, studies will typically include;

Determination of a suitable microbial challenge for use in efficacy studies will typically be performed in 2 or more rounds and success will be measured as an increase or maintenance of burden in target tissues (or a significant signal in a relevant biomarker).

Studies will normally then be performed using a clinically relevant drug (if available) to provide a positive control to compare novel treatments against. On occasion it may be necessary to determine a dose level that is not fully effective for use to study agents that reduce antimicrobial resistance.

Optimisation of existing models may be required for a specific purpose, examples include variations in immunosuppression or pre-conditioning regimens, timepoints of sample collection, biomarker panel, host species and microbial strain. In all instances refinements will be implemented in accordance with the principles of the 3Rs.

Only well validated and clinically relevant drugs will be used in this licence. In all cases historic data will be used to determine the dose and treatment regimens.
Most studies undertaken will be of short duration, lasting less than 7 days.

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

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

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

Other routes of infection may occasionally be used such as skin, bladder, vagina, bone or intestine to model very specific clinical conditions. In these cases, animals will generally be

treated less frequently, usually no more than 4 times daily for up to 7 days, but occasionally may require surgical implantation of catheters into blood vessels to allow for infusion of potential treatments when this is the likely route of treatment in patients.

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

In small numbers of studies animals will have blood samples taken to measure biomarkers of disease in the blood or undergo imaging using non-invasive methods to identify measures that may reduce the numbers of animals required to provide a scientifically valid result.

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

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

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

The most significant impacts on the animals will be a result of the infections. In general infections will cause loss of appetite and so weight loss, reduced activity and changes in core body temperature.
With thigh, bone and systemic infections, pain may also be seen With lung infections an increase in breathing rate and changes in breathing pattern may also be seen. In bacterial models these will be towards the end of a study, usually no more than 24 hours after infection. For fungal and viral models these signs will also occur in the final hours prior the end of the study which may be up to one week in duration

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

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

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

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

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

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

- Mice - 30% Mild and 70%
- Moderate Rats - 30% Mild and 70%
- Moderate Guinea Pigs - 20%
- Mild and 80% Moderate Hamsters - 20%
- Mild and 80% Moderate Cotton rat - 30% Mild and 70% Moderate

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

- Killed

Replacement

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

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

Many of the initial studies performed to help in the validation of antimicrobial targets and assessment of potential medicines now take place in computer, genomic, receptor and cell-based experiments. The hollowfibre infection model can give an intial assessment of how the antibacterial treatments may behave in a living organism prior to being used in clinical trials.

However, even with these advances, computer and cell-based approaches still do not allow the response of the infectious agent to be assessed in a way that reflects the full complexity of an integrated mammalian system, particularly where the immune system response is a significant part of the expected action of the treatment.

The purpose of this project is to optimise or improve infection models, and introduce new infectious disease models to be used on the efficacy licence. The provision of these models will provide the data from a complex, integrated organism to allow decisions to be made on whether to progress potential anti-infective medicines or vaccines to the next stage of development.

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

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

Some or all of computer modelling, genomic, cell-based and non-protected animal approaches will potentially be of value in avoiding or minimising the use of animals in drug discovery and will be assessed prior to undertaking projects using protected animals.

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

For the introduction of new animal models to broaden the Establishment's offer to clients, a literature review will be undertaken to ensure there are no non-animal approaches that could be used to provide the same scientific outcomes.

Why were they not suitable?

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

Reduction

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

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

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

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

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

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

The minimum number of appropriate control groups and control animals are used;

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

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

Small pilot studies may be performed to help design the decision-making studies, including identifying the dose level of infectious agent that gives the best chance of identifying a clinically relevant effect with the minimum possible adverse effects.

Additional control measures to reduce variance such as; animal strain, weight range, supplier will be controlled. Use of standardised protocols and work instructions will provide consistency between operators.

In some situations it may be possible to share control groups with other studies being simultaneously conducted with the same organism.

Refinement

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

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

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

The models include:

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

When required, genetically altered animals that demonstrate no or only Mild signs will be used

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

The use of insect models (waxmoth larvae, Drosophila) for investigation of infectious diseases is described in the literature, however these simple models do not provide sufficient data to enable the development of a thorough understanding of the effects of the potential anti-infective in a fully- integrated organism, which allows the complex interaction of the treatment with many body systems to be assessed prior to moving on the human clinical studies. This means these models are unsuitable for use on this licence.
Most studies will be investigating responses that develop over many hours or days so the use of terminal anaesthesia is not appropriate.

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

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

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

The combination of routes and frequencies of administered substances, including infectious agents, will be performed in the most refined way and not beyond. Further refinement to technical procedures and housing are implemented when they are shown to be beneficial for the animals and will not reduce the quality of the scientific outputs.

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

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

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

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

AWERB and the Named Persons routinely circulate information about the 3Rs and identify opportunities for enhancements during their routine rounds.

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

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

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

23. Neural circuits underlying cognition

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

Hippocampus, Memory, Cortex, Perception, Cognition

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

Our experiments are designed to better understand the neural circuits underlying cognition. Our major focus is on understanding how different brain regions encode information and communicate with each other during behaviour and sleep, and how this information is consolidated into long-term memories. Our experiments focus on measuring and decoding brain activity over active behaviour, learning, sleep, consolidation, and memory retrieval.

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

Why is it important to undertake this work?

This research studies how the brain works at a systems level, specifically how neural circuits interact to compute and process information. This is key to understanding loss of

function in a variety of brain disorders/diseases, such as autism and dementia, and may provide insight into how to help repair the brain.

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

Despite great advances in the field, several questions about how we store memories and how our brain processes information from our surroundings remain unanswered. This project aims to better understand how neocortical and hippocampal regions –two areas known to be involved in memory formation and storage- encode information, how this information is initially stored in memory, and how this memory is subsequently consolidated. Our experiments generally focus on how sensory information is processed by neocortex during behaviour, and subsequently consolidated by interactions between the hippocampus and sensory cortex, during sleep and wakefulness.

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

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

These experiments will be conducted in rodents given the substantial amount of evidence showing that many of the brain structures involved in processing information are preserved through mammalian evolution and are similar in humans. Understanding what are the mechanisms for memory formation and consolidation could be of great value to prevent -or keep to a minimum- the effects that several neurodegenerative diseases, such as Alzheimer's disease, have on memory. Our studies will also provide information about how the brain processes information coming from different sensory modalities (i.e. audition, vision, etc.). Understanding this might contribute to the development of technology designed to help auditory and visually impaired persons.

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

The end goal of our project is to report the findings in an open access peer-reviewed journal, upload our manuscripts to a pre-print server, and deposit our data in an open-access repository. We will promote our work in international conferences, and seek out new collaborations. We will also rely on public talks, social media, and university press releases to help advertise our work to the public. Our experiments are designed to provide knowledge regardless of the outcome of the experiment, and are therefore usually publishable.

Species and numbers of animals expected to be used

- Mice: 7000
- Rats: 700

Predicted harms

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

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

We are using adult mice and rats, as they are vertebrates, have homologous brain regions to humans (e.g. hippocampus), can be trained on complex behavioural tasks, and neural activity can be recorded using chronically implanted electrodes or calcium imaging. We have focused on adult animals, as our research questions target the neural circuits responsible for cognition in the adult brain. Finally, developments in genetic technologies allow the ability to target specific types of cells in the rodent brain, or to introduce genetic mutations that model known correlates of degeneration in humans, a valuable tool in dissecting the function of neural circuits.

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

Our research involves five different types of procedures:

breeding transgenic animals.

behavioural training using positive or mildly aversive stimuli

injecting viral vectors or other tracers into the brain of an anaesthetised animal, to tag neurons or pathways, which allows their interrogation in brain function at a later time point in the awake animal. This is accomplished in a short surgery lasting a few hours, and the animal can be used in behavioural or neural recording experiments afterwards.

chronic recording or modulation of neural activity (either with an implanted microwires, optic fibre and/or a mini microscope). Chronic recordings can take place daily, occurring over several months.

acutely recording and/or modulation of neural activity from a behaving animal. Recordings can be made using an electrode or imaging methods through a small craniotomy, while the head is stabilized with a small headpost. Recording sessions can last several hours (typically 1-3 hours) and can occur over 1-2 week period.

For procedures 3-6, animals will need have an operation, performed under general anaesthetic and using aseptic surgical techniques, to allow experimental interrogation and precise recording of neural circuits. The skull is exposed, grounding and support screws are attached, and small holes are made to allow access to the brain, and a lightweight device is attached to the skull. In some cases, small electrodes or optical guides are implanted in the same procedure. Everything is secured with dental cement (or other appropriate glue- e.g. optibond) before closing the wound. During these procedures we do not anticipate anything more than some minor bleeding which will be stopped immediately during the operation. The animals recover from the anaesthetic in a warmed box, and are then returned to their home cage, where they remain under close observation. The animals may experience some post-operative pain, which will be controlled by the use of pain-killing agents. The severity level of these surgeries is moderate.

After recovery from surgery, the animals are gradually habituated to handling, and head-restraint where it is needed. Animals may be food or water restricted and trained to obtain a food or liquid reward for doing a task. Measurements are made while a sensory stimulus (e.g. a pattern of light) is presented, or while the animal is exploring a small environment. Typically, these measurements take 2-3 hours to complete. In some animals, these measurements will be made once per day over the course of about a week. In other animals we will instead make less frequent measurements (for example, every 2 weeks)

over the course of several months, to track long term changes in the brain. All animals will be killed humanely when these measurements are concluded. The severity level of these procedures will be mild or moderate.

Limits to these procedures are as follows:

Surgery limits

2 major surgeries + 1 minor surgery

or 1 major surgery + 3 minor surgeries

major surgeries include implant surgeries, and are longer in both duration and recovery times

minor surgeries include corrective procedures (e.g. fixing ground wire) and procedures performed on or within the recording chamber (including craniotomies). However, if a minor surgery lasts more than 2 hours (actively under anaesthesia), it will be reclassified as a major surgery.

If an animal has already had two major surgeries, and a minor surgery is reclassified as a major surgery (over two hours), it will be humanely killed.

Common examples:

virus injection (major) + headplate surgery (major) + microelectrode implantation within recording chamber (minor)

Chronic microdrive implantation (major) + corrective procedure (minor)

Headplate surgery (major) + 2 craniotomies for acute recording (minor)

Craniotomy limits

up to 4 craniotomies can be made within a surgery for viral injections or acute/chronic recordings. animals will receive a maximum of 6 craniotomies in their lifetime.

Footshock limits

10 shocks maximum in one session, and 16 shocks cumulatively across animal's lifetime
maximum shock duration is 2 seconds
maximum shock intensity is 0.8 mA Head fixation limits
For daily behavioural training, we train for 1 hour/day, with a limit of 1.5 hr.

For the recordings sessions (max. 6 sessions/craniotomy), we typically record for 2-3 hours, with a limit of 3.5 hr.

Food/water restriction limits for behavioural testing/training

Food restriction- 120 days cumulative (minor food restriction for the purpose of controlling weight does not count towards this total)

water restriction- 120 days cumulative

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

In some animals, a genetic modification to reproduce a potential cause of brain disorder will be introduced. Some of these lines of animals show little evidence of potentially harmful phenotypes at any age, but others show potential for harmful phenotypes at older ages, and we will therefore conduct our experiments before these ages are reached. The severity level of breeding and maintenance of these animals will therefore be mild.

Animals on protocols 3-6 will undergo an aseptic implant surgery under general anaesthesia (AB), typically lasting 2-3 hours (mice) and 4-6 hours (rats) depending on the complexity of the procedure and/or volume of brain tissue targeted. One or more craniotomies will be performed in each animal to allow the stereotaxic targeted injection of a viral vector (or other substances) and placement of an implanted recording/stimulating device above the craniotomy, attached using bone screws (if required) and dental acrylic (or suitable alternative). Larger implanted devices will potentially impede animal movements temporarily, however natural behaviours (e.g. consummatory, grooming, and exploration) are expected to return to normal within a week post-surgery. Surgery may also attach a headplate to allow head-fixed behavioural testing, providing greater control of the location of the sensory stimuli relative to the animal's head.

During this main surgery, may require aspiration of a small region of brain tissue to allow the placement of a GRIN lens above the targeted brain regions (e.g. hippocampus). Additionally, some animals will require the removal of a portion of the temporalis muscle, to allow access to lateral brain regions.

For more complicated viral expression strategies, it will be necessary to inject viral vectors at two time points, and animals will receive two main surgeries (AB), with a minimum of two-weeks between surgeries.

After the recovery period is complete, and animal regains normal behaviour including eating, drinking, grooming, locomotion and exploration, the animal can be (re)introduced to behavioural testing and (optionally) returned to food control or water control.

A small proportion of animals will receive one or more fear conditioning protocols, each providing between 1-10 mild shocks in a given session, and across sessions up to 16 mild shocks cumulatively. Each shock is maximally 0.8 mA for 2 seconds duration, delivered through the grid flooring of the chamber. Fear conditioning is currently the only method of single trial learning, that allows multiple testing sessions with minimal extinction. This is critical in our experiments where we need new memories to form within a training session, and understand how different brain regions contribute to the memory's maintenance and recall.

The majority of animals will undergo behavioural/neural measurements with food/water restriction, for typically for 1-3 months following surgery, although this can be extended based on the experimental constraints and benefit of additional data, as well as the animal's health and implant's condition.
Food/water restriction and fear conditioning will not be both performed in the same animal.

In food/water restricted animals, their food and water intake will be monitored carefully

alongside their weight, but these animals may experience moderate weight loss. If they show signs of ill health or more substantial weight loss, they will be taken off the task and given free access to food and water, and experiments will only be recommenced if they recover well. If they cannot learn the task they will be removed from the study and 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)?

Mild: some (~ 20%) of the animals will only undergo simple behavioural testing.

Moderate: most (~ 80%) of the animals will go through either behavioural testing with a mild aversive component and/or a moderate surgical procedure. In these animals, behavioural or neural measurements are usually made in awake state after recovery from surgery.

Severe: none of the animals are expected to go through a severe procedure.

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

- Killed
- Used in other projects
- Rehomed
- Kept alive

Replacement

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

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

Our experiments aim to understand how memories are formed and the role of sleep in memory consolidation. Because our experiments require a change in the behavioural state of the animal (awake vs. sleep) and testing the formation of memories, we require awake, behaving and sleeping animals. We also need to record neural activity from large populations of single-neurons and be able to manipulate this neural activity to interrogate neural circuits, in order to understand how ensembles of neurons interact together to represent information.

The questions we are investigating require measurements from individual neurons in the brain, which are only possible in very limited circumstances in humans (usually during surgery for epilepsy). These measurements also require the presence of normal sensory inputs and intact brain pathways connecting neurons. These pathways have not yet been replicated in vitro or in silico, and answering these questions therefore makes it necessary to perform experiments on intact animals.

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

We have already considered computer models and non-invasive approaches (fMRI, EEG, and MEG). Computational approaches are already used in conjunction with our experiments to build more accurate models.

Why were they not suitable?

Before we can replace in vivo models with computer models, we require a more detailed understanding of the underlying neural mechanisms of learning and memory. EEG, MEG and fMRI measurements are indirect measures of local neural activity and would therefore not allow us to make the inferences that we need to be able to make (to understand neural circuits at a single neuron resolution).

Reduction

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

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

The number of animals used is based on the number of researchers, the number of animals required for breeding, training, and neural recordings. A typical researcher may use 4-8 rats (and 8-16 mice) per year when performing chronic recordings, 8-16 animals per month for acute experiments, and 12-48 animals per month for behavioural experiments. Transgenic colonies vary in size, but can be as small as 1-2 breeding pairs, or 3-6 breeding pairs if there is a high demand for a particular transgenic line.
We keep the number of animals as low as possible by making large-scale recordings from individual animals. For example recent advances in technology mean that it possible to record electrophysiological or imaging signals from 400-1000 neurons in a single session. As the activity of neurons within the same animal are not independent from each other, however, we will usually use linear mixed-effects models (LMMs) to account for these dependencies when pooling the data from all recordings. LLMs require at least 5 groups, i.e. animals, for a robust estimate of variance across groups. Where we are making measurements of longer-term plasticity or field potentials, we cannot take the same approach. The resource equation and our experience establishes that cohort sizes of about 10 animals provides a generally appropriate number in these cases, at least for planning purposes, where we are making single comparisons (e.g. stimulus-dependence, or strain- dependence).

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

Experiments are designed according to the NC3Rs ARRIVE Guidelines to ensure experimental rigour (blinding, randomisation etc) when possible.

Whenever possible, the animals will be tested for periods of several weeks -as long as the animal is healthy- and thus considerable information is obtained from each animal, minimising the total number used.

Suitable statistical analysis (e.g. ANOVA) and/or the use of linear mixed-effects models

will minimise the number of animals needed to overcome inter-subject variability, and more reliably test the statistically significance of an observed effect. When necessary, we will seek for statistical advice from colleagues and collaborators.

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 the minimum number of mice required to maintain transgenic lines (e.g. two males and one female mouse), to avoid unnecessary production of GM animals. Additionally, transgenic lines are only started when they are needed for a particular experiment. When the number of experimental animals need to be increased due to experimental demand, we will increase these numbers on a temporary basis. Wild type animals resulting from the cross of transgenic lines will be used in other protocols. When possible, we will share common transgenic lines with other laboratories, instead of breeding them ourselves.

Whenever possible, data previously obtained in the lab or outside of the lab will be used to answer new question and we will resort to available databases (e.g. NeuroData without borders: http://www.nwb.org/allen-cell-types-database/).

The recording devices we will chronically implant consist of several electrodes, each of which can record from different neural populations, providing information from a greater number of neurons from the same individual over several weeks. We typically use 24 tetrodes, with which we can record from 50-100 neurons simultaneously (from the hippocampus), which is a twofold increase compared to previous approaches. We plan to keep increasing the number of tetrodes used to record, so that, in a near future, we will be able to collect all the data we need from fewer animals. Calcium imaging and neuropixel recordings also allows the recording of neural activity of large populations of neurons, which will also contribute to reduce the number of animals used.

We usually conduct pilot experiments to make sure that the choice of sensory stimulus that we use is appropriate, and where possible to estimate the variance in the data that we will be collecting. These pilot experiments can often be included at the end of experiments designed to address a previous question, which means that additional animals often do not need to be used. We can therefore adjust our power calculations and related estimates as the experiments proceed, and iteratively reduce the number of animals required to address the questions posed.

The sensory cortices are the best understood of the cortical areas, and there are now several models that start to provide reasonable predictions for the activity of brain cells in these areas. We use these models routinely (e.g. models of receptive fields in auditory cortex) to make predictions about the responses of the nerve cells that we will measure, however most of our questions address the pattern of activity across nerve cells, and the impact of experience on these patterns. The large-scale, dynamic aspects of neuronal activity are not yet well captured by models, but we believe that our and other data will improve this situation over the next few years.

Refinement

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

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

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

We will use rats and mice as mammal models for memory consolidation and sensory (e.g. visual, auditory) processing. Mice give us access to transgenic models that are particularly useful for manipulating and recording brain activity, while rats are able to perform more complex behavioural tasks. The sensory cortex and hippocampal formation is well characterised in rodents, providing a solid basis for studying cortico-hippocampal dynamics underlying memory consolidation and cognition.
Using more primitive animal models (zebrafish, fruitflies, worms) prevents us from investigating a) cortical circuits, 2) REM sleep, and 3) replay using high density electrophysiogical methods. Rodents are the lowest-level animal model system where these questions can be investigated.

Mice will be used over rats for head-fixed acute recordings, and for access to transgenic models that may not be available in rats. Rats will be used instead for larger or heavier chronic implants (needed for studing replay in the dorsal hippocampus) and complex behaviours (or behavioural tasks that are challenging to perform in mice).

In addition, genetic technologies enable the generation of models of brain diseases (such as Alzheimer's Disease) that can recapitulate key aspects of these disorders in humans. Most of our experiments involve performing surgery under anaesthesia to implant recording devices in the brain. Our measurements are then made in the awake state when the animal has fully recovered - the process of making measurements does not cause pain, suffering or distress to the animals.

Head fixation is required for virtual reality experiments (providing control of sensory stimulation during navigation) and acute recording (minimizing the weight of the implant, allowing probe angles that could not be supported in chronic implants).There are no suitable alternatives allowing acute recordings without head-fixation, as the probe must remain stationary relative to the animals brain. Without acute recordings, we would not be able to record in several brain regions simultaneous, when they are located on the lateral portion of the brain (e.g. auditory cortex and ventral hippocampal recordings). We would also be limited in our use of high-density neural probes (neuropixels), due to their size when chronically implanted. Their use limits the number of animals required, by recording more neurons simultaneously in a single recording session.

Fear conditioning is required for single-trial learning. If learning occurs gradually over multiple behavioural sessions, we cannot perfrom causal inteorgation of consolidation mechanisms (e.g. disrupt the hippocampus after learning during sleep and then retest with an intact hippocampus). There is only one other alternative behaviour for single-trial learning- social transmission of food preference.

However, this approach is not applicable to our experiments for three reasons- 1) a subset of brain regions we are studying (e.g. auditory cortex) are not required, 2) the sensory stimulation from the second rodent is not well controlled, and 3) multiple recall tests cannot be performed to study the temporal dynamics of memory. Without the use of fear conditioning, and use of rapid learning tasks, we cannot study the temporal dynamics of

memory consolidation and test how memories become hippocampally-independent.

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

We are studying the neural circuits underlying cognition in rodents, with a translational potential to understand human cognition. The brain regions of interest (neocortex and hippocampus) are only found in mammals (including both humans and rodents). Rats and mice are a less sentient mammalian species, compared to other commonly used species such as ferrets, cats, and monkeys, and have a suitable behavioural repertoire for our experiments. Additionally, the most advanced neural recording methodology available has already been adapted to these species. We are using adult mice and rats, as we are studying cognition in adult animals, and the recording methodologies require a minimum body size to support the device's weight.

The measurements we propose to make are aimed at understanding cognition in the adult brain, when the circuits between neurons have already been largely formed. The brain is still developing rapidly at earlier (e.g. neonatal) stages of development, and this makes interpreting the measurements very difficult. We will make measurements from awake animals because (1) the measurements we propose to make require comparison of activity in the brain before and after exposure to a stimulus (learning), and the timescales of learning extend over days or weeks; (2) anaesthetics have profound effects on brain function, especially in small animals such as rodents, and it is very difficult to know how to interpret measurements of brain function in an anaesthetised state.

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

Post-operative care and pain management: We will minimise pain and discomfort during and after all the procedures performed with animals. Surgeries will be performed under general anaesthesia.

Analgesics and antibiotics will be administered as needed during and after surgery to minimise pain and reduce the risk of infection, respectively. The surgeries will be performed by competent staff to minimise side effects and the length of the surgery. All animals will receive post-operative care to ensure a normal recovery. We closely monitor animals in a separate, heated area, until they are recovered from anaesthesia and are moving freely. We have designed our experimental regimes so that we can avoid surgery on Fridays, and we closely monitor the animals for at least 3 days after surgery, during which time they are also provided with oral pain relief medication. If any animal failed to recover from the surgery, it will be humanely euthanized. We will discuss regularly with the vet if our procedures can be further adapted to improve post-operative care.

Handling and training of animals: We have refined our procedures by placing strong importance on training research staff in better handling, and dedicating several days to initial familiarisation of animals to research staff before surgical or experimental intervention. We have refined our procedures so that instead of holding by the tail we now routinely use 'hand cupping' or where possible 'cardboard tunnels' to move animals from holding cages to transport boxes and apparatus. Where animals need to be temporarily restrained during the course of measurements, we have refined our procedures so that animals are gradually habituated to the apparatus and the restraint (usually over 5 days).

Behavioural testing: External stimuli that could result in stress for the animals (e.g. bright

light, loud sounds, etc.) can be minimised by isolating the behavioural apparatus. If an animal shows signs of stress during the behavioural task, it will be removed from the task until calmed, or completely removed and euthanized if it fails to recover. Fear responses to the behavioural apparatus will be minimised by providing animals time to habituate to the apparatus at the start of the experiment. Food or water restriction will be used only when strictly necessary to enhance animals' learning and motivation, using the minimal restriction required to make the task (and associate food/water reward) sufficiently motivating for the animal. We will be vigilant of new published methods for positive reward, in search of a suitable replacement. In a subset of animals, we will conduct automated behavioural training within their home cage, allowing the animals to train when they want to during the day or night, without leaving their cage and avoiding any contact with the experimenter. This approach maximises the rate of learning while minimising stress in the animal. Where head restraint is required to avoid animal movement, distress will be minimised by gradual adaptation.

Environmental enrichment: We have refined standard cage design so to provide greatly increased space between the floor and the ceiling, substantially reducing the possibility that implanted animals become entangled. This advance means we can now also routinely enclose running wheels in the mouse cages without entanglement.

Implantable devices: We will use low-weight micro-drives (allowing natural behaviour of the animal within a week post-surgery) to minimise discomfort after implantation. We are constantly working in developing lighter drives in order to minimise discomfort and stress for implanted animals.

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

We will follow the Laboratory Animal Science Association guidelines (https://www.lasa.co.uk/wp- content/uploads/2017/04/Aseptic-surgery-final.pdf) for the application of aseptic techniques during surgery.

We are also referencing the following guidelines:

Experimental design: https://arriveguidelines.org/arrive-guidelines

Experimental procedures: Barkus et al. (2022) Refinements to rodent head fixation and fluid/food control for neuroscience. Journal of Neuroscience Methods, 381: 109705. https://doi.org/10.1016/j.jneumeth.2022.109705

Breeding / colony management: https://nc3rs.org.uk/breeding-and-colony-management, and https://nc3rs.org.uk/3rs-resources/breeding-and-colony-management/worked-example-intermittent- breeding

Animal monitoring: https://nc3rs.org.uk/3rs-resources/grimace-scales

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

The PPL holder will attend at least one online 3R seminar each year, to keep up to date with new advances in the 3Rs (https://nc3rs.org.uk/events)

Receiving regular newsletter updates from N3CR on advances in 3R practices.

The PPL holder will have regular discussions with our NACWO and NVS, on new ways to refine our methods, according to new advances in the 3Rs (e.g. newsletter, seminars)

24. Mechanism of stem cell homeostasis, cancer and tissue 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

Stem cell, Cancer, Regeneration, Therapy

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim of this project licence is to characterise the cellular and molecular mechanisms regulating stem cell fate decision, and their corresponding roles in tumourigenesis and tissue repair, which will help develop new cancer treatment strategies.

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

Why is it important to undertake this work?

The human body is made up of trillions of cells that together form various organs to carry out their unique functions. The growth and maintenance of each organ is supported by the stem cells in the resident tissues, which will respond to damage for tissue regeneration. This is achieved by delicate control of signals given to the stem cells for their fate decision (expand, mature, dormant or die).

Dysfunction of these cell regulations can lead to uncontrolled expansion (cancer) or catastrophic loss (organ failure) of stem cells. The purpose of this project is to understand how fate decision is controlled by specific genes and genetic pathways in both normal (healthy) and abnormal (trauma, inflammation, cancer and aging) situations. These genetic controls of stem cells are often similar in different organs/systems. The main system we use is the gut, which is one of the fastest regenerating tissues.

Stem cell fate decision, tissue repair and cancer are closely linked, while the underlying molecular control is not fully characterised. Our aim is to identify the key genes and their related signalling pathways involved in the stem cell control of normal, injured (e.g. irradiation, inflammation) and cancerous tissues, with the emphasis of finding new drug targets to improve treatment of cancer and inflammation. We are also interested in understanding how aging affects the highly regenerative intestinal stem cells and the consequences such as inflammation and cancer. In addition, we aim to develop tissue engineering strategies to grow a small bowel in a dish to treat intestinal failure patients caused by trauma or congenital disease. Transplanting the engineered grafts in mice will be an important preclinical step to test the safety and engraftment efficiency in animals before going to patients.

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

Our project is likely to advance the basic understanding of how human body works by stem cell fate decision, to benefit diagnosis of genetic diseases, to inform clinical treatment, and ultimately to improve cancer treatment by providing options of new targeted (personalised) therapy. We will share our results with other researchers in the form of peer-reviewed original research articles and reviews in specialised scientific journals. Negative data will also be published to reduce replicate experiments and therefore further use of animals by others in the future.

The intestinal tissue engineering objective will further generate potential patient-specific functional and transplantable intestinal grafts using patient-derived organoids, which offer treatment hopes to intestinal failure patients.

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

Academic and clinical research scientists and students of stem cell and cancer biology would benefit from the knowledge/resources generated in this project. Novel drug targets and tissue engineering or regeneration approaches may also be discovered for developing new therapeutic strategies.

Therefore, ultimately patients with various types of diseases such as cancer and trauma may benefit from these novel treatments. Intestinal failure patients may also benefit from the potential treatment alternatives using our laboratory-grown intestinal grafts.

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

We aim to disseminate our findings to scientists in our own and other fields, as well as more widely in the public domain, by presentations at national and international meetings and publications in high- impact journals. We also work alongside the Translation team who support collaborations with industrial and clinical partners, which will enable more efficient translation of our research findings into the clinical settings. We have been

working on tissue engineered intestine with our clinical collaborators over the last 6 years. Their clinical expertise will certainly help translate our results into the clinic.

Species and numbers of animals expected to be used

- Mice: 24,000

Predicted harms

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

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

The purpose of this project is to understand specific genes and genetic pathways regulating stem cell fate decision in both normal (healthy) and abnormal (trauma, inflammation, cancer and aging) tissues. Therefore, we need to use mice as a model for our project, because their physiology, anatomy, metabolism and pathological manifestations better resemble those in humans compared to the features of other commonly used animal models, such as nematode (C. elegans), fruit fly (Drosophila melanogaster), or zebrafish (Danio rerio). Mice also have shorter latency of tumour development compared to higher vertebrates, which is the ideal physiological animal model for cancer research studies.

Different life stages of mice are chosen to address specific research questions. For instance, embryo, neonate, juvenile, adult and aged mice will be used for phenotyping studies to understand stem cell regulation at various life stages, whereas study of cancer progression and treatment will mostly involve juvenile and adult animals. Transplantation work will be performed on adult mice that are bigger and more resilient to the surgical procedures.

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

Animals will be used to study stem cell fate decision, tumour development and regeneration. We will focus on studying the intestine, but other organs such as liver and stomach may also be included for analysis since they are closely related physically and metabolically to humans.

For stem cell studies, artificial deletion or expression of genes will be used to study the role of specific genes in intestinal stem cell maintenance. Animals will be monitored and will be culled at assigned time points or at approved humane end point (in case animals display clinical symptoms). Short-term (1 to 30 days) or long-term (1 to 15 months) induction will be performed depending on the research question. Their tissues will be dissected to examine the molecular and cellular mechanisms of the processes driving stem cell maintenance and fate decision.

For tumour studies, animals will be used to develop heterotopic (under skin) or orthotopic (intestine) primary tumours. For advanced tumour models, metastasis may be observed (e.g. in liver), as it happens in human cancer. The tumours will either appear spontaneously due to inherent genetic alterations or be induced by the administration of either viruses or plasmids that will carry either enzymes or genes enabling such genetic alterations or by a transplantation of cancerous cells or tissue pieces. Tumour induction

time varies depending on the tumour models. E.g. subcutaneous injection of aggressive cancer cell line (such as B16F10) will form tumours within days and animals may need to be culled within 2 weeks. On the other hand, spontaneous tumour models, such as Apcmin/+ mice, will start to develop intestinal tumour at around 6-8 weeks' time and will experience clinical signs at around 5-8 months. After tumour induction, animals will undergo different experimental procedures, such as blood sampling for monitoring, injection with labelling agents that would facilitate visualisation of certain types of cancer cells or drug treatment. Animals may undergo imaging to monitor tumour growth by using both modalities that are used in humans, e.g., ultrasound, endoscopy or MRI. All animals will be closely monitored for tumour size and/or clinical sign and will be culled according to experiment timelines or at humane end point whichever comes first.

For regeneration studies, intestinal tissues will be injured by chemicals, irradiation or genetic approaches and will be closely monitored to study tissue regeneration. The methods used are well established injury models with well-defined progression and monitoring. Typical irradiation study will be relative short-term, where mice will be collected for analysis after 1-7 days, although longer time points (months) may be considered when assessing the return of homeostasis after regeneration. All animals will be closely monitored for clinical signs and will be culled according to experiment timelines or at humane end point whichever comes first. Our lab also develops tissue engineering approach for innovative organ replacement therapy. The engineered grafts will be transplanted to animals (under skin, under kidney capsule or onto the intestine) to test the growth and survival in animals.

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

Most of our regulated experiments are of the mildest severity and concern the breeding and observation of genetically altered mice and/or minimally invasive procedures such as administration of substances by injection. Adverse effects are neither expected nor seen in all but a very few of these cases. In some cases (particularly for tumour studies), the animals will develop tumours, which can be associated with weight loss, signs of discomfort and slowing down of the normal activity. However, the procedures will never exceed the moderate severity level. Any animal approaching severity limits will be culled by approved humane methods, and all animals subject to a procedure will eventually be culled and their tissues will be dissected and used for analysis.

In mice genetically predisposed to develop cancer spontaneously, tumours typically develop in the period between 6-8 weeks time; in some cases, up to 12 months. For transplantation models, the time frame between tumour transplantation to the humane endpoint is shorter, typically between 3 to 12 weeks. Thus, some of the adverse signs mentioned above will develop over a shorter period of 1–2 weeks for transplantation model. In all cases, tumour burden will be limited to the minimum required for a valid scientific outcome. Animals may display tumour ulceration, laboured respiration, or persistent diarrhoea, but they will be immediately culled after any of these symptoms is observed. The animals will also be monitored for weight loss and body condition score, and mice dropping below the set criteria (e.g. 15% weight drop) will be immediately culled to prevent excessive suffering.

For injury studies, we will use sub-lethal dose of irradiation or chemical treatment. Typically, tissue damage will be induced within days, and animals are expected to make a full recovery soon after. The animals will be monitored for weight loss and body condition

score to prevent excessive suffering before experimental end points (15% body weight over 72 hours or 20% weight loss of the initial weight for injury studies).

Animals that will undergo surgical procedures may experience mild to moderate pain immediately after the surgery (within hours) and they will be given analgesics to minimise post-surgical pain.

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

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

For the mice used for stem cell studies (~30% in total), the severity will be mild. For mice that will be developing tumours in the course of the project and/or will undergo surgeries (~50% in total) the severity will be moderate. Approximately 30% of these animals will be used for tumour and other tissue collection after being killed by a Schedule 1 method or after a non-recovery procedure under general anaesthesia. The rest will be either administered different gene/protein modifying or therapeutic agents, and/or placed on modified diets. In all of the cases the overall severity will not exceed moderate level. For the rest of the animals, which can be either genetically modified but without any obvious clinical signs or wild type and/or receiving infrequent injections, the severity will be mild. The severity of breeding animals will be mild but the majority 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?

To identify mechanisms of stem cell fate decision, tumour growth and regeneration in physiological condition, which are meaningful for human clinical studies, we need to use animal models because they resemble human physiology and they develop tumours similar to human ones. In addition, the effect of local and systemic drug treatment and dietary manipulations can only be meaningfully studied in vivo with the complete tumour microenvironment (e.g. immune cells and blood circulation).

With the use of patient-derived organoids, we have recently developed innovative intestinal tissue engineering strategies to reconstruct patient-specific functional and transplantable intestinal grafts using patient-derived organoids, which offer treatment hopes to intestinal failure patients. We hope that these laboratory-grown organs could offer a safe and longer-lasting alternative to traditional donor transplants in the future. Testing the safety and functions of these lab-grown organs in animals is an important pre-clinical step prior to translation in the clinic.

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

Our lab is specialised in generating in vitro organoid models to study intestinal stem cell

biology, cancer modelling and drug screening as well as organ reconstruction. Organoid is a powerful model that allows us to perform preliminary functional screening in vitro to identify important candidate genes involved in stem cell fate decision and cancer progression. In addition, we have recently developed innovative intestinal tissue engineering strategies to reconstruct patient-specific functional and transplantable intestinal grafts using patient-derived organoids, which offer treatment hopes to intestinal failure patients.

Evidence will be collected from initial screening studies using in vitro cell culture/organoids/tumourspheres model systems (derived from either healthy or diseased tissues) to shortlist candidate genes that show functional significance in the in vitro systems. Extensive literature review will also be performed to validate the relevance and importance of the shortlisted candidate genes. In addition, we will use non-regulated procedures to collect expression data from fixed non-GM mammalian tissues and functional/expression data from genetically and/or environmentally manipulated in vitro system. For examples, expression of gene of interest can be tested using existing wildtype intestinal tissues in the lab or interrogated in the transcriptomic datasets generated by our lab or publicly available. Those generated data sets will be analysed to identify the most promising candidates for functional validation in animals.

Why were they not suitable?

Cell fate decisions and tumour development in the animal or an organ take place within a complex environment, where events intrinsic to the cells are influenced by a variety of extrinsic signals. The latter can involve molecules that can act locally or over considerable distances (such as growth factors and hormones), and which may originate from neighbouring cells (e.g. immune cells), or from anywhere within the body (or even be from the external environment such as microbes in the gut).

Moreover, most tissues develop in a complex way in three dimensions over time in a carefully orchestrated manner, and require blood vessels and nerves to operate. It is not yet possible to recapitulate all of these parameters in vitro. Therefore, although some aspects of certain cell fate decisions can be studied in vitro, and we both use and develop such approaches, it is generally essential to study them in animals (as a minimum to judge the suitability of in vitro systems to give meaningful information). This is particularly true of the complex systems and processes we investigate.

Reduction

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

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

The number of animals to be used have been estimated on the basis of the previous 10 years of work of my lab and the current landscape of projects of the lab going forward.

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

Our experiments are designed to use the minimum number of animals required to give robust answers with the use of statistical methods. The efficiency of animal usage is maximised in consultation with animal technicians by careful control of breeding to match research needs with respect to numbers, phenotypic uniformity and health. Wherever possible, we will limit the use of genetic models (that often require many generations breeding) using orthotopic transplants of labelled cells and treating the mice with chemical agents either to block immune-system components or to generate tumours. In addition, we will also take advantage of the online tools, including the NC3Rs Experimental Design Assistant to help us with experimental designs.

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

We test methods and reagents in vitro whenever possible prior to their use in animals.

We always confirm the importance of the genes of interest in “organoid culture” (3D stem cell culture that consists of organ-specific cell type) in vitro before testing them in animals.

We use efficient methods to generate and maintain genetically altered animals, and make use of sperm and embryo freezing to avoid keeping the strains as live animals when a particular study is finished.

We will make optimal use of several tissues, fluid and cell types per individual mouse and will provide the other affected tissues to appropriate scientists so that they do not have to breed mice specifically for their 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 have been selected for the majority of this work as it is an appropriate model for providing insights into human diseases and it is the species in which reliable transgenic and knockout technologies are most advanced. We will induce gene deletion or cell labelling of genetically engineered mice to study stem cell fate decision. For tumour studies, we will use spontaneous genetically engineered models of cancer alongside more controlled experimental models of inducing cancer, including transplantation techniques. The choice of the models depends on the research questions and the complexity of the projects. Genetically engineered mouse models (GEMMs) are more physiological tumour models, but often take longer time and more animals to establish. For example, when testing the tumourigenic role of a new candidate gene, GEMMs will be used if we are testing early tumour development with less genetic modification (e.g. only mutation in Apc gene), whereas transplantation of tumour cells or organoids combined with gene editing will be more appropriate and efficient if more advanced/genetically more complex tumour

models (e.g. mutations of Apc, Kras, p53 and Tgfbr2) are tested. Our work focusses mainly on intestinal cancer but may also study secondary tumours such as liver metastasis. We minimise the use of surgical methods, wherever possible, by employing endoscopy-guided injection techniques which drastically reduces the suffering experienced by the animals. For regeneration studies, mice will be treated with chemicals, irradiation or genetic approaches to induce transient tissue damage and will be closely monitored to study tissue repair. The methods used are well established injury models with well-defined progression and monitoring. Animals are expected to make a full recovery within a week or two. For safety and functional studies of the engineered grafts, we will transplant the grafts to mice to monitor their engraftment, survival and functions.

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

Less sentient animals (such as nematodes, flies or zebrafish) that could be considered for these types of studies lack the organ and physiological complexity needed for translation to humans. This complexity needs to be recapitulated to make the investigation of tissue/tumour microenvironment possible. For instance, flies do not have adaptive immunity that is crucial in humans to generate antibodies, whilst fish lack a comparable microbiome as in mammals. The requirement here is to have a physiology which is as close as possible to humans, and only mammalian organisms have similar complex immune system, hormonal infrastructure, basic metabolism and microbiome as humans. Therefore, mice would be the most appropriate animal model for our project.

For animals at a more immature life stage, although some objectives in this project may benefit from studying younger mice, pups or embryos (objective 1 to understand intestinal stem cell development), these are not suitable for some other goals, especially when studying tumour development and tissue transplantation.

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

We choose well-established protocols known to have minimal harmful effects whenever possible.

To minimise stress during breeding and maintenance, we will follow best practice guidelines and follow local refinements of husbandry.

Whenever practical, we prefer to make genetic alterations that are inducible, so that the animals do not show a phenotype until expression of the candidate gene or a deletion is induced.

For manipulations, we will adhere to local and national guidelines that aim to minimise suffering. If insufficient information is available, new manipulations will be pre-screened in small-scale pilot studies to obtain indications of the minimal dose and exposure time that is likely to be effectively, thereby minimising any potential suffering.

In all surgery, analgesia will be provided according to contemporary best practice and advice from the NVS/NACWO. Good aseptic surgical techniques, heat and fluid therapy will be provided as necessary.

We have recently established the endoscopy-guide orthotopic transplantation of cancer cells/organoids, which will minimise animal suffering caused by surgeries.

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

Unless otherwise specified, the work in this project will be designed using the principle outlined in PREPARE guidelines for planning animal research and testing (1) and in the LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery.

With regards to the experiments in cancer models, we will adhere to the Guidelines for the Welfare and Use of Animals in Cancer Research (2) and will monitor body condition according to scoring systems developed by Ullman-Culleré and Foltz (3). In addition, I will also follow the latest advancements in relevant fields, by attending conferences, reading journal articles, and collaborating with experts in these areas, to ensure that the experiments will be conducted in the most refined way.

Smith, A. J. et al. PREPARE: guidelines for planning animal research and testing. Lab Animals 52, 134–141 (2018).

Workman, P. et al. Guidelines for the welfare and use of animals in cancer research. Br J Cancer 102, 1555–1577 (2010).

Ullman-Culleré M.H. and Foltz C.J., Lab Anim Sci. 1999;49(3):319-23

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

We regularly follow the websites and receive updates on advances in the 3Rs from within our establishment from NC3Rs and NORECOPA. I will also keep following up the latest publications in the fields as well as attend courses and seminars.

25. Manufacture and development of a coccidiosis vaccine

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

Vaccine, Coccidiosis, Poultry, Global, Chicken

Animal types	Life stages
Domestic fowl (Gallus gallus domesticus)	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 manufacture and development of Chicken vaccines to meet the Global demand for an effective anti-coccidial vaccine in compliance with the European Pharmacopeia Monograph.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 provide a vaccine to prevent coccidiosis in chickens

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

The primary benefit of the use of birds is to produce Eimeria oocysts for the manufacture of approximately 6.5Billion doses of coccidiosis vaccine over the duration of the 5-year License.

Continued supply of Coccidiosis vaccine for Poultry, will lead to vaccinated birds not being affected by coccidiosis or the associated morbidity, mortality and reduced weight gain, promoting better welfare for the vaccinated chicken.

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

Poultry Industry will be provided a vaccine throughout the 5 years. Globally economic costs of approx.

$3bn/year, a small percentage of which are treated with vaccination programs.

Coccidiosis is a protozoic parasite that infects the intestines of the animal it has “chosen” for its host. The protozoa parasites in the intestinal lining, damages the organ, rendering it unable to absorb the nutrition chickens need to thrive.

The licence enables approximately 1 Billion doses per year to be supplied globally, the birds vaccinated are not affected by coccidiosis or the associated morbidity, mortality and reduced weight gain, promoting better welfare for the vaccinated chickens.

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

Collaboration with, Poultry Veterinarians, Coccidiosis experts and colleagues globally, some of whom are responsible for manufacturing similar coccidiosis vaccines outside of the UK.

Species and numbers of animals expected to be used

- Domestic fowl (Gallus gallus domesticus): 760,800

Predicted harms

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

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

GMP (Good Manufacturing Practice) Manufacturing Licence issued through VMD details the use of four to seven weeks old SPF origin (specific pathogen free) chickens.

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

Disease free eggs are incubated on site, healthy chicks are reared to four weeks of age, in cage systems with gridded floors including an area of solid flooring. The alternative to this is birds are supplied at approximately four weeks of age from an accredited breeder.

The birds are then transferred into dedicated Production cages, also with gridded floors and perching is available for enrichment. The birds are then dosed with a suspension of oocysts either orally or in their feed. For production of some of the strains, where improved yields can be achieved, an immunosuppressant is added to the drinking water.

Faecal material is collected for recovery of oocysts at a designated point when the output

is high.

Birds at the end of the collection period (faecal collection periods differ per species 24-72hrs) are humanely culled immediately after the collection period has finished (a maximum of 12 days post infection). The manufacturing License dictates the collection period for each species, any modification will lead to Regulatory changes to the dossier. The period of collection is determined by the oocyst output pattern and any extension would provide minimal gains.

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

Due to the attenuated nature and minimal dose of the vaccinal challenge organisms, no clinical signs of infection are expected, birds drink, feed and grow normally throughout. Should any adverse signs occur the birds are humanely killed.

Although the birds have been acclimatised to the gridded floors, they are continually monitored for the occurrence of foot injuries, and if discovered the birds 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)?

Mortality is not expected as part of the procedure, however a small number typically in the case of the breeds used currently in production may suddenly and unexpectedly die or be euthanased in extremis. Mortality greater than 1% post challenge will be reported under PPL standard condition 18 and investigated.

Mild - applicable to any bird that receives infective material via feed, oral gavage or receives drinking water that has been treated with an immunosuppressant, while being housed on gridded floors.

Moderate applies to any birds that are culled due to exhibiting signs of ill health that could be potentially due to procedural harms.

Severe applies to any birds that are found dead and their death is potentially due to procedural harms. Based on previous years return of procedure:
Mild ~99.6% Moderate ~0.2% Severe ~0.2%

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

- Killed

Replacement

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

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

Coccidia are host-species specific and do not complete their life-cycle in vitro and therefore use of chickens is required in order for the pathogen to complete its life cycle. A review of the potential methods of oocyst production in the manufacture of the Paracox vaccines has confirmed that it is not economical or efficient to use either monolayer cell cultures (only two Eimeria species will potentially develop), or embryonated chicken eggs – a limited number of species would reliably develop and the yields would be very low compared with the current production system. To produce the significantly high numbers of the oocysts required for the commercial manufacture of the vaccine it would not be feasible or economical. (Williams 2007). Current literature searches confirm that chickens continue to remain the only feasible option for commercial manufacture as all the species will develop to the oocysts.

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

Recent and extensive online searches have been made for possible non-animal methods (NAMs) to evaluate for potential use and possible replacement within the vaccine production process.

Databases of animal alternatives organisations in EU and US e.g. https://ntp.niehs.nih.gov/data/index.html and https://caat.jhsph.edu/about/ have been searched with scant results of significant value regarding recent 3Rs implementation that have been documented.

The EURL ECVAM search guide https://op.europa.eu/hr/web/general-publications/publications has also been investigated with a comparable paucity of results and also the UK NC3Rs page on alternatives https://nc3rs.org.uk/3rs-resources/search?topic%5B0%5D=504has been investigated with similar results.

European colleagues are working on the Eimeria project and looking into alternatives, initially focusing on the vaccine potency test, and are investigating development of a viability assay. They are also actively working on a qPCR for identity and quantification, this potentially could be used as an alternative potency test and have been looking into growth of Eimeria on cultured cells i.e., they are searching for an alternative vaccine solution that doesn’t require live oocysts.

Monolayer cell cultures

Why were they not suitable?

Monolayer cell cultures are not capable of producing all eight strains due to complex life cycle of oocysts.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 capacity of the Primary Production Unit is 10,000 multiplied by 16 Lots/Year annually for 5 years life of the PPL.

Typically we need to use up to 140,000 chickens to produce 1bn doses of coccidia vaccine per year. Birds used for development purposes area small percentage, based on 2022 data this equated to less than 1%.

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

Extension of the collection period for the one of the eight vaccine component strains has demonstrated a 13% reduction in birds required to achieve the same volume of doses. The manufacturing License dictates the collection period for each species, any modification will lead to Regulatory changes to the dossier. The period of collection is determined by the oocyst shedding pattern, any extension will provide minimal gain. Protocol 2 enables studies to be performed for further refinements to reduce the number of birds required to be used.

Through several improvement steps in the clarification of another strain, sterility failures have been significantly reduced. Continuous improvement in Operational planning allows maintenance of a high level of antigen utilization and reduction of the number of birds used per dose; this is currently over 7000 doses of final vaccine produced per bird used in production.

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

Monitoring of oocyst outputs to ensure optimal infection of each bird on a Lot by Lot basis. Collection periods are optimised for volume of recovery of oocysts.

The use of an immunosuppressant (corticosteroid) administered in the drinking water and the extension of the patent period for two species of Eimeria, results in substantially more oocysts to be harvested from birds used. It is used at a low and continuous dose and has no perceivable adverse effects.

Refinement

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

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

Chickens are the target species for the host-specific coccidia, and alternative methods are not capable of growing sufficient doses of the required Eimeria species.

Dosing of some working seeds in the feed versus oral gavage has reduced animal

handling and optimised the uptake of coccidia and improved yields.

Animal well-being is maximised by providing the good environmental conditions, such as ensuring birds have perches available in each cage.

Doses are measured carefully for optimum results ensuring the wellbeing of the chickens, oocyst concentration in the dose is initially reduced to account for viability as the age of seed increases.

The oocysts are attenuated, causing minimal harm to the birds.

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

Target species is required to be used as coccidia is species specific.

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

Refined by infection via feed opposed to orally.

Gavage and Cloacal routes are limited to Seed Production, the cloacal route is to be investigated for sub master and master seed infections of 2-6 birds at a time.

The process of antigen production starts with a frozen volume of a specific oocyst Master Seed (MS). Each vial of MS contains a predetermined number of organisms stored in liquid nitrogen. Typically, 2 birds are infected with one vial of MS to produce the first passage of Working Seed (MS1). This is then passaged further to increase the number of oocysts. MS1 Working Seeds are then passed through typically 8 birds to produce the MS2, then further passed to produce MS3 and MS4 Working Seeds.

Sub-Master Seeds (SMS) are produced to replenish the MS stocks and are derived from MS4 Working Seeds. The first passage from a SMS is called MS5 and is treated the same way as an MS1 Working Seed. The MS5 Working Seed can be passaged up to 3 times to produce the MS6, MS7 and MS8 Working Seed. When sufficient numbers of oocysts are produced, the Working Seed is converted into Production Seed by sterilising the material and making it up to a specific concentration of oocysts/ml.

Seed production is limited to approx. 5% of the birds. Infection periods are limited, and birds are culled immediately after the collection period, maximum duration from infection to collection is 148 hours.

Twice daily inspections are routine within all areas of Production. If any birds are deemed unwell, the birds will be culled and additional checks will be implemented by the NACWO and PPL holder.

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

Recent and extensive online searches have been made for possible non-animal methods (NAMs) to evaluate for potential use and possible replacement within the vaccine production process.

Databases of animal alternatives organisations in EU and US e.g. https://ntp.niehs.nih.gov/data/index.html and https://caat.jhsph.edu/about/ have been searched with scant results of significant value regarding recent 3Rs implementation that have been documented.

The EURL ECVAM search guide https://op.europa.eu/hr/web/general-publications/publications has also been investigated with a comparable paucity of results and also the UK NC3Rs page on alternatives https://nc3rs.org.uk/3rs-resources/search?topic%5B0%5D=504has been investigated with similar results.

Searches have included:

Shirley, M. W. (1989), Development of a live attenuated vaccine against coccidiosis of poultry. Parasite Immunology, 11: 117–124.

Williams, R.B. (2003). Anticoccidial vaccines: looking back, looking forward. Zootecnica International, 25 (7/8): 52-57.

European Pharmacopeia monograph: Coccidiosis vaccine (Live) for Chickens (07/2008:2326).

Peek, H. W. and Landman, W. J. M. 2011. Coccidiosis in poultry: anticoccidial products, vaccines and other prevention strategies. Veterinary Quarterly 31:143-161.

H. J. van der Sluis , R. M. Dwars , J. C. M. Vernooij , and W. J. M Landman. 2009. Cloacal reflexes and uptake of fluorescein-labelled polystyrene beads in broiler chickens. Poultry Science 88 :1242– 1249

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

Communication with Colleagues and different departments within the network. Coccidiosis forums and 3 Rs website. Periodic searches completed for potential non animal methods.

26. Defining chlamydial pathogenesis and control of ovine enzootic abortion and Q fever in small ruminants

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Chlamydial pathogenesis, Pregnancy, Vaccination, Small ruminants, Reproductive diseases

Animal types	Life stages
Mice	pregnant, adult
Sheep	adult, pregnant, juvenile
Goat	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 enable greater understanding of disease progression in small ruminants of ovine enzootic abortion (OEA); immunity during ovine pregnancy and design of single and combined vaccines to reproductive diseases principally OEA and Q Fever.

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

Why is it important to undertake this work?

OEA is the most common cause of diagnosed ovine abortion in the UK. The disease is difficult to diagnose particularly during early stages of disease using conventional serum-based tests. The current range of live vaccines available are not completely effective at reducing burden and available inactivated vaccines have lower reported efficacy. There is a desire to develop a new protective vaccine so that could be deployed alongside a rapid penside test to allow the separate identification of diseased/infected from vaccinated sheep (DIVA). Such diagnostic and control tools are long term goals of this project.

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

The overall outputs of this project will lead to a refined subcellular vaccine to OEA. It is anticipated that this vaccine will be commercialised with an industrial partner. It will also generate knowledge of vaccine induced immunity to OEA that will lead to peer-reviewed publications. Progress towards a next-generation vaccine to OEA utilising recombinant antigens or recombinant viral vectors is anticipated. Such use of recombinant technologies in vaccine manufacture could refine the manufacturing process and facilitate a move away from the whole pathogen-based live and killed vaccines- reducing issues related to their associated safety and length of immunity.

Information obtained could be used in the development towards a combined multi-pathogen vaccine for reproductive diseases in small ruminants. The development of a Point-of-Care (POC) test could facilitate more efficient on-farm OEA diagnosis.

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

In the short term, the research group, those interested in reproductive health of small ruminants in the establishment and elsewhere may benefit from the outputs. The farming community would directly benefit from commercialisation of POC tests and a safer efficacious vaccine to OEA at the end of this project. Improved diagnosis and management of chlamydial disease affecting reproduction in small ruminants may potentially have an impact worldwide. Better disease control will lead to lower numbers of chlamydial abortion and improved animal health and welfare through a reduction in environmental contamination and pathogen transmission. Reduction of infectious *Chlamydia* in the environment will directly reduce the chance of animal to animal, and animal to human disease transmission.

Collectively, these improvements lead to better farm productivity and efficiency, positively impact farm sector sustainability and reduce greenhouse gas emissions.

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

The outputs of the work will be maximised through a variety of ways including collaboration with industrial partners for commercialisation of POC tests and vaccines. Practical knowledge derived from the project objectives will be disseminated to stakeholders through peer-reviewed publications, newsletters and policy documents. It is anticipated that success in development of diagnostic POC and prototype viral vector platforms could be adapted for use for other small ruminant diseases affecting reproductive health. Characterisation of a safer vaccine to OEA alongside other vaccines to reproductive disease may provide information on any interference of vaccine-induced immunity and

inform on development potential of multi-pathogen reproductive disease vaccines.

Species and numbers of animals expected to be used

- Mice: 300
- Sheep: 800
- Goats: 100

Predicted harms

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

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

Use of mice will provide a surrogate pregnant model of chlamydial abortion. This will enable the accelerated refinement of vaccine formulations (number of antigens and delivery mechanisms). This will directly reduce the number of sheep used in experimental trials.

Female sheep of less than 2 years old of high health status will be required for setting up natural host models of OEA and the assessment of prototype vaccines for ability to prevent disease.

Female goats less than 2 years old will be brought in for the assessment of vaccine-induced responses to commercial and prototype vaccines. Additionally, goats would provide a source of cells for the assessment and characterisation of immunological reagents and assays in small and large ruminants.

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

Mice will be bled by venesection prior to immunisation through the oral, subcutaneous, intra-muscular, intraperitoneal or intranasal routes. A second booster immunisation may be given at least one week later and a further tail bleed taken after two weeks. The female mice will be synchronised for oestrus and then mated using the Whitten Effect. Mated, pregnant mice will be singly-housed and then experimentally inoculated with *Chlamydia abortus* (*C. abortus*) via injection through the intra-peritoneal cavity route at day 11 of pregnancy. The mice will progress to the end of gestation- pre-term abortion in most cases or delivery of live pups. Surviving pups and adult female mice will be euthanised by an approved Schedule 1 method. These animals will be subjected to five procedures and the duration of the study will be around 10 weeks- subject to efficiency of Whitten Effect mating.

Sheep will be bled by venepuncture to the jugular vein at defined intervals for the duration of pregnant sheep *C. abortus* vaccine-challenge studies. Animals will be given up to three immunisations through the oral, subcutaneous, intra-muscular, or intranasal route separated by a three week interval. The sheep will be mated and then experimentally inoculated with *C. abortus* via injection through the subcutaneous route at day 70 of pregnancy. (If oral route of inoculation is used, these sheep would be experimentally infected prior to pregnancy). The studies are likely to run for around 35 weeks and involve approximately 13-16 procedures.

Sheep and/ or goats will be bled by venepuncture to the jugular vein at defined intervals for the duration of immunogenicity studies. Animals will be given up to three immunisations through the oral, subcutaneous, intra-muscular, or intranasal routes separated by a three week interval. The studies are likely to run for around 12 weeks and involve approximately 10-12 procedures.

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

Female mice may be transiently affected by the immunisation and/ or experimental infection. Tail bleeds will cause mild discomfort. Mice may develop a short lived fever following infection with live, defined organism. Clinical signs of illness can be divided into the following three categories: (a) presumed febrile response, as determined by the appearance of the coat; (b) Dehydration, determined by weight loss; (c) General demeanour. These will be monitored and clinically scored as outlined in protocol 1. Should the accumulative score threshold be reached, then the mice will be killed using a Schedule 1 approved method.

If a mouse develops a swelling or abscess at the site of injection with antigen in adjuvant that is considered to be causing undue suffering then it will be killed by an approved Schedule 1 method.

The pregnant female mice following experimental infection, non-immunised mice and some immunised are expected to abort prior to full term of pregnancy which would have a moderate but transient impact.

Mice under distress have been known to cannabalise foetuses and/or young pups. No additional signs of distress are generally observed.

Following experimental infection of small ruminants with *Chlamydia abortus* (*C. abortus)* a fever may develop at or around the third day and have returned to normal by the tenth day. Animals would be expected to continue to eat and drink normally during this time. Following infection of pregnant ruminants *C. abortus* may establish in the placenta and foetus. In a proportion of cases the foetus or newborn animals will die. This abortion event would have a moderate impact on the ewe of a transient nature. One would not expect the ewe to show other significant clinical signs of illness beyond the act of abortion and then proceed to ignore the non-viable foetus post parturition. The ewe will not show any other visible signs of distress and only show expected mothering behaviours towards surviving lambs.

Expected severity categories and the 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 **Protocol 1** mice and sheep may experience the following severity levels:

Mice: Around 50% of all mice will experience moderate severity including all pregnant mice infected with *C. abortus* infectious challenge with no prior vaccination and under 1% of vaccinated and challenged mice.

Under 50% of all mice will experience mild severity including pregnant mice: with prior experimental vaccines and infected with *C. abortus* challenge plus unvaccinated, unchallenged animals.

Sheep: Up to 50% of all sheep will experience moderate severity including pregnant sheep inoculated with infectious *C. abortus* challenge material only and some with prior vaccination.

Up to 50% of of all sheep will experience mild severity including pregnant sheep: given *C. abortus* challenge and prior vaccination(s), delivering live lambs and not exhibit behavioural changes at inoculation, challenge or lambing; and unvaccinated, unchallenged sheep.

For **Protocol 2** sheep and goats may experience the following severity levels:

Sheep: Up to 10% of all sheep will experience moderate severity. Up to 90% will experience mild severity.

Goats: Up to 10% of all goats will experience moderate severity. Up to 90% will experience mild severity.

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

- Kept alive
- Killed

Replacement

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

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

Chlamydial species are obligate intracellular bacterial pathogens that require a live cell to replicate. There are no cell-free systems for the growth of these pathogens that have been successfully developed. Whole chlamydial cell-based vaccines and challenge inocula require cell-based production methods. The commercial OEA vaccines (produced by MSD Animal Health and CEVA Animal Health) still use fertile hens' eggs for production. We have developed alternate cell culture systems for the preparation of the in-house vaccine material, which has removed the use of eggs for this purpose.

Immature fertile hens' eggs (within the non-regulated first two trimesters of gestation only) are required for the production of live *C. abortus* stocks of high titre and quality. The consistency achieved by this production method, to date, has ensured a greater chance of success in the experimental animal models. This makes the results of any challenge experiment more certain, reducing the risk of experimental failure and the need to repeat the work in additional animals. However, recent advancements in cell culture and purification techniques, in our research group, have dramatically improved titre of recoverable viable organisms using *in vitro* cell culture. It is anticipated that production of challenge inocula will move over from egg-based production to cell culture-based protocols once validation of the new methodology has been completed early in the course of this programme of work.

The use of live animals (sheep) is also required to conduct pathogenesis studies and test vaccine efficacy in the natural host where the assessment of clinical disease and the whole body response to infection is required. For OEA, animals are infected through the oro/nasal cavity and infection is observed in the uterus/placental unit of pregnant animals close to the end of gestation.

Every effort has been made to reduce the need for animal studies using *in vitro* assay systems e.g. the use of *ex-vivo* T-cell screening assays for identifying potential vaccine candidate antigens prior to conducting evaluation in live animals. Such assays developed at Moredun have helped to assist in the identification of antigens that could elicit a protective immune response, thus replacing or minimising animal usage.

Animals are only used after *in vitro* alternatives have been thoroughly investigated and exhausted, or that the use of alternatives will likely mean that the outputs will not be achievable, which would then result in experiments being repeated and require a greater number of animals being used.

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

In vitro alternatives are always thoroughly investigated and exhausted first. Recent work to develop *in vitro* enhanced cell cultures (organoids) in ruminants may allow assessment of protective antigens prior to *in vivo* trials immunogenicity or efficacy (vaccine-challenge) studies. This will involve further development which is planned for during the lifetime of this licence.

Why were they not suitable?

The enhanced cell cultures require further development to enable the creation of experimental *in vitro* models that will allow direct interaction between mucosal cells and immune cells in ruminants to model key aspects of OEA progression.

Reduction

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

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

The number of animals have been estimated based on the experience of the research group, information from expert collaborators/ scientific literature and the planned funded requirements for the programme of work to follow. It is necessary to conduct a pilot study to re-initiate the pregnant mouse *C. abortus* challenge model. This model will be conducted at least twice in this programme of work to reduce down the number of antigens and delivery mechanisms used for subsequent assessment in efficacy studies in sheep.

There will be one sheep pathogenesis study and two vaccine-challenge studies. Information on the size of the groups used for the sheep challenge studies will be discussed below.

In addition, it is anticipated there will be at least one immunogenicity study for sheep/goats. The group sizing will be based on numbers used in previous immunogenicity studies in sheep and in goats at MRI and our collaborators.

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

I am aware of the Establishment 3Rs Committee and are aware of their training and Continued Professional Development opportunities and will engage this committee during the experimental design phase. In addition, I have consulted various online sources of information including: Understanding Animals in Research, Laboratory Animals Science Association website and Norway's National Consensus Platform for the advancement of "the 3 Rs" (Replacement, Reduction, Refinement) in connection with animal experiments (Norecopa) website to help reduce the numbers used in this project.

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

The number of animals used in studies will be kept to a minimum. Results from the mouse model will refine the number of antigens and delivery mechanisms to be tested in the natural sheep host, resulting in a reduction in the number of sheep used in experimental trials. The numbers used in both the mouse and sheep models will be informed by past experience, existing datasets and current expert project collaborators at the Establishment together with independent statistical advice provided to the Establishment by the statistical advice provider. All experiments require approval of the Establishment Ethical Review Committee, who specifically evaluate number of animals used in relation to the cost:benefit balance and minimising animal use.

Refinement

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

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

Great efforts have been made to avoid the use of sentient animals whenever possible.

Advances in the use of cell culture for the growth of chlamydial organisms have reduced the requirement to use embryonated hens’ eggs. The methodology has been refined to remove the use of sentient embryonated hen's eggs within the final trimester of gestation. It is used to produce challenge inocula of sufficient pathogenic quality and high titre. This method will be phased out for challenge inoculum production once an evaluation with cell-culture derived inocula has been undertaken in the early phase of this project.

Routine growth of chlamydial organisms for molecular and cellular studies and for the production of vaccine material will be conducted *in vitro*.

Immunogenicity studies will be conducted in sheep or goats to assess prototype and commercial vaccines to OEA and another reproductive disease of ruminants, Q Fever. This will refine the number of prototype vaccines that will go forward for efficacy testing.

Animal studies will only be conducted when there is sufficient evidence that *in vitro* research has reached an end-point and that further progress will only be achievable through the use of animals, where we can assess vaccine success through a reduction in the number of abortions occurring. All animal studies will be subject to approval by the Establishment's Animal Ethics Committee prior to commencement. All animals will be closely monitored daily to ensure their continued health and welfare. Any animals found to be suffering as a result of procedures specified in this licence will be treated immediately by one of the on-call veterinarians. If necessary, the animals will be removed from the study and euthanised humanely by Schedule 1 method.

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

The aims of this project are to assess and develop vaccines to a disease that affects pregnancy. Mice and sheep are necessary to reproduce aspects of the complex processes following *C. abortus* infection that require the pregnant animal. *C. abortus* causes a latent non-productive infection in non-pregnant sheep. In non-pregnant mice, they naturally resolve infections with little clinical manifestation of disease.

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

I will liaise with the Establishment 3Rs Committee on future experiments to determine if there are additional enrichment measures that can be undertaken for the mouse, sheep and goats for planned *in vivo* trials.

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

I have reviewed and will apply the current PREPARE 2.0 and Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidance on best practice and reporting on animal experimentation. This will ensure that the studies are conducted in the most refined manner.

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

I currently receive email updates from the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) on matters relating to Animal Experimentation. I will regularly visit the website NORECOPA for the Planning Research and Experimental Procedures on Animals: Recommendations for Excellence (PREPARE) guidelines; relating to best practice for animal research and testing. I am also aware of the Establishment 3Rs Committee and receive regular newsletter updates from it. I will attend the 3Rs Experimental Design and Ethical Review FRAME Course and future Experimental Design Assistant training courses.

27. Exploring bioelectronic approaches to nervous system restoration in large animals

Project duration

5 years 0 months

Project purpose

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

Key words

Bioelectronics, Neurological restoration, Brain injury, Spinal Cord Injury, Peripheral nerve injury

Animal types	Life stages
Sheep	adult
Pigs	adult
Minipigs	adult

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim is to create about three small electronic devices that can be placed inside the body (bioelectronic devices) to help fix lost nerve functions due to injuries in three parts of the nervous system: the brain, the spinal cord, and the nerves outside the brain and spinal cord (peripheral nerves).

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

Why is it important to undertake this work?

This work is essential because it tries to create new ways to treat nerve-related diseases and problems that have been hard to solve before, like paralysis, Parkinson's Disease, epilepsy, and injuries to the brain or spinal cord. Also, these devices will help us learn more about these diseases and might help find even more new treatments.

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

Devices: This project will lead to the creation and improvement of three devices designed to work with nerves in humans, which will:

1. Give detailed, long-lasting recordings of nerve structures, helping us understand different nerve-related conditions and diseases better and create focused treatments.

Fix nerve functions by reconnecting and reshaping damaged nerve pathways. Help us learn more about how electronic devices can work with our nerve system.
Data: The devices will gather lots of information from both the main part (central) and the outer parts (peripheral) of the nerve system. This data can be used, for example, to help create computer programs and teach special math formulas (algorithms) for more advanced research and medical uses.

Sharing what we learn: We will tell other scientists about our discoveries by writing articles for important science magazines that other experts check (peer-reviewed journals) and talking at science meetings (conferences). We will also share with the public to help people understand science better and make them more aware of nerve-related diseases.

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

Nerve-related disorders affect up to 1 billion people all over the world, with about 6.8 million people dying from them each year. Brain injuries, spinal cord injuries, and injuries to nerves in other parts of the body are big reasons for disability and not being able to do everyday tasks.

In the short term, our technology will help us learn more about the complicated connection between electronic devices and the nerve system. In the medium and long term, the devices we create to work with nerves will have many benefits for patients suffering from different nerve-related conditions:

Allow us to record nerve activity and control how nerves work. Fix broken nerve connections in people with injuries or damage.

Give new ways to treat patients with hard-to-control epilepsy, long-lasting pain, Parkinson's, and Alzheimer's disease.

By working on these problems, our project could help make life better for millions of people who have trouble with their nerves because of diseases or injuries.

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

To get the best results from our work, we will:

Use strong research methods and work together with experienced researchers.

Share our findings (including unsuccessful approaches) in important medical journals that other experts read and talk about early results at big conferences around the world.

Keep good relationships with top groups in the field of electronic devices that work with nerves, so we can share new knowledge effectively.

Involve people who can make decisions early in the process and work to quickly turn our technology into something that can be used in real life.

Watch and measure how well our research is doing and change our approach if needed to get the best results.

Species and numbers of animals expected to be used

- Sheep: 100
- Pigs: 250
- Minipigs: 100

Predicted harms

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

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

We have chosen to use bigger animals, like adult sheep and pigs, for these reasons:

Early testing: We've already tested the three devices we made at our place in the lab, on the computer, and in smaller animals like mice and rats. These tests showed that the devices work well in recording nerve activity and helping to control how nerves work.

Limits of using mice and rats: While mice and rats are helpful for testing in the early stages, they are quite different from humans, so the results might not work the same way in people.

More similar to humans: By testing our devices in bigger animals like adult sheep and pigs, we can get more useful information about how safe and effective our technology is. This will also help us make the devices, surgical methods, and how we put the devices in the body better for people.

By using bigger animals with bodies more like humans, we can learn important information for making and fine-tuning these devices, making sure they are safe and work well for people.

The animals we've chosen for our experiments are adult sheep, pigs, and minipigs. Here's why:

Sheep: Sheep's brains are very similar to humans in some ways. This makes them useful for our experiments, where we are testing devices that interact with the brain. Sheep are also generally easy to work with and are very docile, which helps make sure they stay comfortable and stress-free during our work.

Pigs: Pigs are often used in medical research because their bodies respond to treatments in ways that are similar to humans. For our experiments, we're particularly interested in their nerves. Pigs' nerves are a lot like human nerves, which is important for our research into devices that can help control nerves.

Minipigs: Minipigs are a smaller kind of pig that we'll be using in later, long-term experiments. Like regular pigs, they're similar to humans in important ways. Because they're smaller, they're easier to handle, but they still give us the benefits of working with a larger animal that's a lot like a human.

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

In this project, animals will be given medicine to make them fall asleep and not wake up again (non- recovery). Small devices will be put into their brain, spinal cord, or nerves in other parts of their body, including muscles. After the experiment is over, the animals will be killed in a kind and humane way, following a method that causes the least suffering.

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

In this project, animals will be given medicine to make them fall asleep and not wake up again, making sure they don't feel any pain or discomfort (non-recovery). Since the experiments are non-recovery, there won't be any long-lasting effects on the animals. After the experiment is over, the animals will be killed in a kind and humane way, following a method that causes the least 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)?

Animal Type	Category (Expected severity)	Proportion
Sheep	non-recovery	100%
Pigs	non-recovery	100%
Mini-Pigs	non-recovery	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?

We're working on a project that studies diseases that affect the entire nervous system, just like they would in people. To do this, we need to work with a full, working nervous system. This allows us to record, control, and stimulate nerve activity in a way that is close to what happens in humans. It's important for us to be able to work directly with nerves that are still inside the body and working normally because of the kind of devices we're testing.

There are other ways to study nerves, like working with them in the lab outside of the body (in vitro), or using computer simulations (in silico). However, these methods don't completely recreate the complex interactions and processes that happen inside a living, working nervous system. They also can't give us all the information we need about how safe our devices are, how well they work, or how they interact with living tissue.

Because of these reasons, we need to use animals for our research. This will help us get reliable data that can be used to develop and improve treatments for nerve diseases in people.

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

We thought about using the following non-animal options for this project: Lab-grown models, like human stem cells; Computer simulations; Organoids (small groups of cells that act like an organ); Studies of dead humans and large-scale studies of people.

Why were they not suitable?

The non-animal alternatives we considered were not suitable for our project for the following reasons:

Lab-grown models, like human stem cells: These models don't have the same complexity and organization as a whole nervous system, so they can't copy the interactions and processes we need to study.

Computer simulations: Although they're helpful for designing and improving devices at first, computer simulations can't fully tell us how safe, effective, and compatible our devices will be in a living being.

Organoids (small groups of cells that act like an organ): Even though organoids can act like some parts of the nervous system, they don't have the full complexity and interactions with other systems that we find in a living animal. This makes them less suitable for our project.

Studies of dead humans and large-scale studies of people: These methods can give us important information about the structure and function of the nervous system, but they don't let us see how our devices work, how safe they are, and how effective they are in a living being.

Due to these limitations, we determined that the use of animals is necessary to achieve the aim of our project.

Reduction

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

design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

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

Our estimation of the number of animals needed for this project is based on several factors. Pigs will be the main animals we use because they're often used in research for small devices that go inside the body, and they're similar to humans in many ways. We anticipate using 250 pigs in our experiments.

Sheep will be used sometimes because they're a good choice for studying how computers can connect to the brain. Based on our experience and the specific requirements of the experiments, we estimate that 100 sheep will be needed.

Minipigs, which are smaller pigs, are used when we want to study something for a long time and gather early information. We have allocated 100 minipigs for these purposes.

The total number of animals is based on at how much work we're doing now with rodents, how much our lab can do, and our experience with similar research. We've thought carefully about these things to make sure we use the smallest number of animals possible while still reaching our project's goals.

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

What did you do during the planning stage to use fewer animals in this project? - While planning our experiments, we took several steps to use as few animals as possible:

Collect more data from each animal: We carefully set up our experiments to get the most information we can from each animal, making sure we only use the smallest number of animals needed to get important and reliable results.

Improve our tests: We always try to make our experiments better, which helps us need fewer animals to get the information we want. For example, we do a lot of computer simulations, which helps us test fewer things in living animals.

Test more than one device on one animal: Whenever we can, we will test more than one device on a single animal. This means we need fewer animals for each experiment.

Use a special online tool: We use the NC3Rs Experimental Design Assistant, a website that helps us plan strong and repeatable experiments. This tool helps us use fewer animals in our research.

By doing these things, we work to use as few animals as possible while still reaching our project's goals.

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

Besides planning our experiments well, we will do several other things to make sure we use as few animals as possible:

Test things on a small scale first: We will try out new methods and steps in small experiments before doing them on a larger scale. This way, we can make sure we don't use more animals than we need to.

Use computers and data analysis: We will use advanced computer programs to help us look at our results and make computer models. If we can, we will use special techniques to get more information from our data, which means we need fewer animals.

Share samples and work together with other researchers: We will work closely with other scientists and share the samples we get from the animals after the experiments are done. This can help save animals in other studies and make better use of our resources.

By doing these things, we try to use as few animals as possible and make sure our research is ethical while still reaching our project's goals.

Refinement

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

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

During this project, we will use pigs, minipigs, and sheep as our animal models. To minimize pain, suffering, distress, or lasting harm, we will implement the following measures:

Anaesthesia: Animals will be placed under anaesthesia (medicine that causes loss of sensation and consciousness) during the device implantation process (putting devices into the brain, spinal cord, and peripheral nerves), ensuring they do not experience any pain or discomfort. The anaesthesia will be provided by a trained Veterinary Anaesthetist or a competent PIL holder.

Non-recovery procedures: Animals will not be allowed to regain consciousness following the implantation procedures, eliminating the possibility of experiencing postoperative distress or suffering.

Humane euthanasia: After each session, animals will be euthanized (put to death humanely) using an overdose of anesthesia, ensuring a swift and humane end.

By implementing these methods, we aim to minimize any negative impacts on the animals involved in our research while still effectively achieving our project's goals.

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

We cannot use less aware animals or animals at a younger life stage because our devices are intended for people. Although earlier testing allowed us to avoid using large animals, our project has now reached a stage that requires testing on fully grown, working, and

healthy nervous systems similar to those of people.

Using larger animals like pigs, minipigs, and sheep, which are more like people in terms of their body structure and how their body works, allows us to better apply the results to tests involving people.

Furthermore, to minimize any potential suffering, all animals involved in the project will be given anesthesia so they don't wake up, ensuring they do not experience pain or distress during the procedures.

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

Before surgery:

We will give animals time to get used to their new surroundings after they arrive and spend time with them (e.g., 15 mins twice daily) to help them feel less stressed before giving any medicine.

We will make the pigs' living area more fun by adding things that let them do natural behaviors like digging and looking for food, and prevent them from getting bored.

If we need to hold animals still during any procedures, we will teach them to be comfortable with it so they feel less stress.

We will take steps to make sure the animals don't feel too stressed while they're being moved.

During surgery:

We will pick the right animals: Healthy animals that are the best fit for the tests we need to do.

We will use the right kind of sleep medicine: Making sure the sleep medicine we use is the best choice for the type of animal and the procedure we're doing.

We will keep an eye on the animals while they're having surgery: Watching their heartbeat, how fast they're breathing, blood pressure, and other important signs, as well as how they react to things during surgery. This will let us change the sleep medicine if we need to, making sure the animal is asleep but not too much, which can help avoid problems and animals dying when they shouldn't.

If we need to keep an animal by itself for some time, we will explain why, how long, and what extra care and fun things we'll give them during that time.

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

We will follow these published guides to make sure our experiments are done in the most refined way: The Animals (Scientific Procedures) Act 1986

The Code of Practice for the Housing and Care of Animals Used in Scientific Procedures

The Guidance on the Operation of the Animals (Scientific Procedures) Act 1986

The ARRIVE Guidelines

The NC3Rs Guidelines: The National Centre for the Replacement, Refinement, and Reduction of Animals in Research (NC3Rs)

The PREPARE guidelines

Species-specific resources from the Norecopa website for animals in research (https://norecopa.no/species/farm-animals/) and minipigs (https://norecopa.no/species/farm- animals/minipigs)

Pig Housing & Handling resources from the NA3RsC

We will also monitor and have ongoing discussions with the NVS (Named Veterinary Surgeon) and NACWO (Named Animal Care and Welfare Officer) about using non-UK resources to make sure we follow the best practices for animal care and handling.

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

We are committed to providing the best care for the animals we work with and will actively stay up to date on improvements in the 3Rs by:

Subscribing to the NC3Rs newsletter (https://www.nc3rs.org.uk/news) to receive regular updates on new ideas and research related to the 3Rs.

Making sure that all team members know and understand the idea and values of the 3Rs. We will use online training resources such as the Procedures With Care website (https://researchanimaltraining.com/article-categories/procedures-with-care/) and the Experimental Design Assistant website (https://www.nc3rs.org.uk/3rs-resources/search?topic%5B0%5D=497) to teach new team members and remind everyone of the importance of the 3Rs in our research.

Reviewing our research plans every three months to see if we need to make any changes or updates. Improving the way we work with animals is an ongoing process, and we know that we might need to take more steps as we learn more.

28. Fish tracking in the Bristol channel

Project duration

5 years 0 months

Project purpose

- Basic research
- Protection of the natural environment in the interests of the health or welfare of man or animals
- Research aimed at preserving the species of animal subjected to regulated procedures as part of the programme of work

Key words

Rays, spurdog, herring, Acoustic tags, Marine distribution, Marine development, Marine Protected Area

Animal types	Life stages
Atlantic herring	adult

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The overall aim of this work is to collect data on data poor commercially exploited species (4 species of ray, Spurdog and Atlantic herring), to assess migration patterns, survival, and seasonal residence in the Bristol Channel area. To do this we propose to tag the fish with acoustic pinger tags and follow their movements for one or more years with a tracking network of approximately 100 passive acoustic receivers distributed throughout the Bristol Channel. The project will build on our existing Bristol channel passive acoustic receiver array which has already been used to successfully track multiple species, including sea trout, Atlantic salmon and twaite shad.

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

Why is it important to undertake this work?

The project aims to provide data for a number of data poor species of elasmobranchs and Atlantic herring, which will contribute to future management plans and associated management measures, understanding of stock identity and migration patterns, and inform regulatory assessment of major marine developments. Target species comprise both recreationally and commercially exploited species including elasmobranchs (Spurdog, Thornback ray, Spotted Ray, Blonde ray and Small eyed ray) and Atlantic herring. The lack of data on marine migration and distribution of these species and hence potential impact of policy measures and developments, has impaired the ability of policy makers and developers to assess impacts and propose suitable mitigation, compromising marine licence applications and potentially putting the fish populations at risk.

The work will provide evidence to support policy developments including identification of key areas for protection measures and quota setting. It will also provide evidence for impact assessments; the abstraction being built for Hinkley point C, and proposed tidal lagoons in Swansea Bay and elsewhere in the Bristol Channel are major developments where this work has significant current application.

The project will also collect and supply data to other researchers tagging fish in the Bristol channel and surrounding area.

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

Quantitative data on distribution and residence times of tagged species in key 'hotspot areas'

Qualitative data on coastal distribution and migration paths in the wider Bristol Channel area.

Marine survival rates.

Comparison of similarities and differences in migration patterns between species; predator prey associations.

Project report(s) and publications on all species

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

The project will provide evidence which will have long term benefits to policy makers and regulators by identifying marine areas / habitat of particular importance to commercially and recreationally exploited species. This is important in the development of both marine protected areas and quota setting.

Support for Marine Renewable development is an important element of Welsh Government policy. However lack of data on key marine species is recognised as a strategic information gap for Marine Renewable energy (see for example ref 1 below), and other developments such as major abstractions for power generation, by both regulators and industry .

Developers, regulators and policy makers will be provided with valuable data as the study progresses. Information specific to the area will benefit local regulation (Natural Resources Wales) and inform evaluation of current and future development proposals as well as aiding understanding of the effectiveness of the MPA. The information will therefore

provide both short and long term value.

Results describing migration and behaviour patterns in inshore areas will have wider utility and will benefit assessments by regulators elsewhere in the UK (Environment agency, Marine Management Organisation, Natural England and Marine Scotland). The value regulators place on this data is reflected in financial commitments (tag and receiver purchase) to help support the work.

Marine Energy Wales. Tidal range: Critical Evidence gaps and how to address them. Workshop report, March 2022.

http://www.orjip.org.uk/sites/default/files/u53/ORJIP%20OE%20MEW%20Tidal%20Range%20Workshop%202022%20V1.pdf

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

The project is collaborative with regulators, charter vessels and local fishermen. We are already working closely and collaborating with Natural Resources Wales, Natural England, Environment Agency, and the Devon and Severn IFCA, as well as local fishery interests.

Information will be shared with all the partners through regular update meetings with data presentations (at least 2 per year), and the production of interim project reports (which partners will be consulted upon and which will be published on one or more partner websites). A final project report will be published and made publicly available (see above). The more important results will be published in peer reviewed journals. We would also expect to provide information regularly during the course of the project to the wider scientific and development community through conference papers and peer reviewed publications.

Species and numbers of animals expected to be used

- Other fish: No answer provided

Predicted harms

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

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

All the species selected are data poor, commercially exploited species, which are important to both commercial and charter fishers in the Bristol channel. Very little is known about their distribution / migration paths within the Bristol Channel, limiting the ability of policy makers and regulators to formulate appropriate fishery management measures, or to assess impacts of major developments.

We are looking at adults because they are the commercially exploited stage and because they are the most valuable life stage for future reproduction and spawning.

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

Fish will be captured using rod and line or netting / research trawling techniques. Methods

used will minimise the risk of damage during capture.

They will be anaesthetised and tagged with an acoustic tag through an incision approximately 1cm long (or less). The incision will be closed with a dissolvable suture and covered with a suitable covering to provide a waterproofing barrier to protect the wound during the initial stages of healing. Analgesia will be applied as appropriate.

After recovery from anaesthesia they will then be released to continue normal lives.

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

Experience has shown that fish rapidly recover from anaesthesia and surgery and are not expected to suffer any lasting long term harm as a result of the procedures under this protocol being carried out.

The procedures carried out in these protocols will be done under general anaesthetic (with the exception of the ray species) and therefore fish will be subjected to no more than mild stress as a result of capture and handling. There may be some mild post-operative discomfort, but experience of staff carrying out the work will ensure that fish are only released when they are recovered and able to swim actively.

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

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

We expect the severity to be moderate for Spurdog and herring, which will undergo anaesthesia and surgical procedures for coleomic insertion. For ray species we expect the effect to be mild. The tag will be externally attached to a Petersen disc. These are attached by passing a stainless steel wire through the wing which will be a rapid and relatively minor process, without the need for anaesthesia and any associated effects. The tagging approach is intended to minimise discomfort or damage to the fish.

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?

The project aims to look at the behaviour and distribution of elasmobranchs (Spurdog, Thornback ray, Spotted Ray, Blonde ray and Small eyed ray) and Atlantic herring, in the wild, alongside anadromous species (salmon sea trout and twaite shad (ongoing work, already licenced)) in order to gain information to inform our understanding of multi species habitat use and seasonal distribution in the Bristol channel area. This will inform the development of fishery management policy and management measures, as well as

informing impact assessments of specific development areas. There are no practical alternatives to generate this data.

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

Movement and behavioural modelling has already been used for some of this work. However all such work has to be grounded in real observed data to demonstrate that the assumptions underlying the models are realistic.

Why were they not suitable?

The use of models to predict impacts requires real data to demonstrate that the assumptions underpinning the model and the predictions are realistic. Our work to date has already demonstrated that for existing assessments (eg twaite shad presence), without validation by real data, such models may be grossly flawed. This can have important ecological implications for the species as well as significant economic implications for fishers and developers.

Reduction

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

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

We need to use sufficient fish to provide robust estimates of marine distribution to inform models which can then be used to predict impacts without the requirement for further experiments using live fish. We have drawn on a combination of movement data from our existing studies of twaite shad and sea trout, as well as literature studies on similar species, to inform our sample sizes.

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

We have used information from studies of similar species to set initial samples sizes. We are planning to use an adaptive experimental design which will be updated on an annual basis to determine numbers deployed in each year enabling the number actually tagged to be the minimum necessary to achieve the objectives of the study. In year 1 up to 200 ray (from 4 species), 100 spurdog and 100 herring will be tagged. We expect to get multi year data from many of the fish.

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

Ongoing review of data, developing computer models which can be used in subsequent studies to reduce requirements for similar work.

Refinement

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

Which animal models and methods 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 capture and tagging methods we are using with acoustic tags are well established, and are designed to allow the fish to return as rapidly as possible to normal behaviour with minimal long term effects.

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

Our objective is to observe and understand the behaviour and distribution of the fish under natural conditions at sea. This cannot be achieved by other means.

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

Choice of tags

We are using the smallest tags available consistent with the objectives of the project, including tag life and tracking in the marine environment.The tags we are using are specifically designed by suppliers (Innovasea or Thelmabiotel) for work with the species and life stages we are using. They are tough and smooth to minimise any issues if ingested by a predator.

Tagging and recovery procedures

The anaesthesia techniques we are using are well established. Aseptic surgery techniques and single use scalpel blades and suture needles will minimise risk of infections.

Each incision will be covered with a suitable gel to provide a waterproofing barrier to protect the wound during the initial stages of healing. Sutures will be checked prior to transfer into recovery and holding tanks. Analgesia will be applied as appropriate.

Fish will be recovered and will not be released until they are capable of holding station and swimming against the current.

All procedures will only be performed by suitable trained and qualified individuals (ie PIL holder; training and competency records will be kept by the NTCO).

For the ray species we have considered the options of internal tagging and external tagging. Both approaches will require fitting a Petersen disc in the wing (for internal tagging an external mark will still be needed to ensure commercially caught fish do not enter the human food chain during the anaesthetic withdrawal period. The external mark will also enable reporting of commercial captures).
As a Petersen disc will be required in both circumstances, and can provide an effective external attachment point for the tags, we have opted for external tagging of these

species.

Humane end-points and limits of severity

If internal damage to organs were to occur during surgery, the fish would not be allowed to recover and would be euthanized by a schedule 1 method.

If fish fail to recover from anaesthesia they will be euthanized by a schedule 1 method

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

There are a number of published studies using these tags and techniques. However methods evolve continuously and we continue to share experience with others to develop best practice through conferences and direct conversations with other groups, including NRW, Hull International Fisheries Institute, Plymouth University, the Atlantic Salmon Trust, and the Game Conservancy Trust. All the above are undertaking current licenced work with these species and our approach and protocols seek to take the best from each, consistent with our objectives.

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

We will continually review the literature. We will attend conferences, and continue to network with others to share and learn from further developments, both as research understanding of the field develops and to improve our tagging methods to minimise any potential adverse effects. Where appropriate we will update our protocols and methods.

29. The physiology of mammalian eggs and early embryos.

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

Egg, Spermatozoa, Fertilization, Reproduction, Calcium

Animal types	Life stages
Mice	adult, pregnant, juvenile, neonate, embryo Hamsters (Syrian) (Mesocricetus auratus)

Retrospective assessment

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

Objectives and benefits

Description 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 improve our understanding of how the sperm activates the egg during fertilization. This will help design new ways of improving IVF treatment and develop novel forms of contraception.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 addresses two different problems with regards to fertility. One is concerned with promoting fertility, the other with inhibiting it.
In developed countries there has been a major decline in the number of children born per

women and many women are choosing to try to conceive over the age of 30 when their natural fertility is in decline. Many couples face problems in conceiving. In Vitro Fertilization, IVF, is the main option for couples facing persistent problems with conception and there are more than 2.5 million treatment cycles of IVF across the economically developed world. Since its introduction in the 1980s IVF treatment has given rise to well over than 8 million babies, however the overall success rate of IVF (babies born per treatment cycle) has remained the same, at around 1 in 4, for the last 20 years. The number of eggs that are successfully fertilized is a significant determinant of the overall success of the IVF procedure. The events of fertilization can also affect the success rate of later steps in development. In this project we aim to provide new insights into the fertilization process in mammals and to develop new ways of stimulating fertilization and development.

In many developing countries many women and girls face a significant and opposite type of problem. This is one of accessing safe and effective contraceptives. A second part of this project is designed to help design new short acting, non-hormonal contraceptives that will allow women to space out pregnancies and control their own fertility. This is contributes towards a long-term project which is part of a United Nations Sustainable Development Goal (3.7.1).

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

The outputs will be in the form of scientific research articles. In addition it is possible that there may be a patent application filed which will be based partly upon work in this project. This, and other lines of research, could lead to product development with commercial companies that are making products in the IVF industry, or making novel contraceptives.

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

There will be different groups of people who will benefit from this research. The research community will benefit from studies that advance our understanding of the process of fertilization in mammals. These will be short term and realised during and shortly after the project.

Other beneficiaries will be couples who are attending clinics for IVF treatment. We hope to develop improved methods for stimulating eggs to begin development and some of these methods could be developed towards the end of the project or in the 5 years following the project.

Some aspects of the project on non-hormonal contraceptive could also benefit women throughout the world who wish to use contraceptives. This will be a long-term benefit that may take about a decade to be realised after the end of the project.

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

The research articles will be published in peer reviewed international journals that are open access. Outputs will also involve review articles, talks at national and international conferences, and on occasion talks to the media. Any data or research tools generated will be made available to other researchers in the field upon request. This work involves a collaboration with a local IVF clinic from where we source human eggs that have failed to fertilize after mixing or injecting sperm into eggs. This collaboration involves talks at local meetings of reproductive biologists and at meetings of the IVF staff in the clinic. The

applicant has links with people the IVF industry who will be contacted for commercial development of any patented ideas. The applicant will also engage in a collaborative network that is developing non hormonal contraceptives.

Species and numbers of animals expected to be used

- Mice: 3500
- Hamsters (Syrian) (Mesocricetus auratus): 200

Predicted harms

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

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

We study the mechanisms of fertilization and early development in mammals with relevance to human fertilization. To do this we will use eggs and sperm from mice and hamsters. Mouse eggs and embryos provide an effective model of human fertilization and early development. Mouse eggs have very similar metabolism to human embryos and the same culture medium can be used in mouse and human eggs. The similarity is illustrated by the fact that all the quality controls for plasticware and culture medium used in IVF clinics is carried out by culturing mouse embryos. All the key discoveries made in IVF were pioneered first in mouse, or other mammals, before being tested in human eggs and embryos. This includes the discovery of the sperm protein that activates the egg, called PLCz1. Reproductive biology research in mouse is also particularly effective because the success rates of embryo development are high, and because there are genetically modified strains available to investigate the roles of specific proteins. We intend to use male mice lacking a functional PLCz1 gene.

Hamster eggs have also proved to be an effective model for human fertilization. The genetic models are not available in hamster, but hamster eggs have a particular feature that is not seen in mouse eggs. If the outer layers are removed from the hamster egg, it can be fertilized by sperm from many other different species, including humans. This feature appears to be unique to hamster eggs. The so called ‘hamster egg penetration’ test was used for many years as a way of assessing the fertilization capacity of human sperm. This test is legal within the UK and can be licenced by the Human Fertilisation and Embryology Authority. We will develop a more advanced version of this test to the effects of inhibitors on fertilization by human sperm.

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

In order to produce a large number of eggs, female mice and hamsters must undergo 'superovulation'. The typical experiment involves two successive injections of hormones that are given about 48 hours apart. This is part of the superovulation procedure. After another 15 hours the mice or hamsters are killed and the eggs collected from the oviducts. For the breeding of genetically modified mice there are no specific procedures carried out other than ear biopsy for genetic testing.

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

The superovulation technique is well established and has been used in mice and hamsters for many decades, and there are no anticipated adverse effects. Animals will be injected with hormones using standard routes (subcutaneous or intraperitoneal). They will experience mild, transient pain and no lasting harm.

The breeding of mice lacking PLCz1 is not associated with any known harm. The only phenotype that is known is reduced fertility of male mice. Animals will experience mild and transient discomfort from blood sampling for genotyping.

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

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

All procedures to be carried out are considered mild and no adverse effects are anticipated.

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

- Killed

Replacement

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

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

To study the process of fertilization we need to use sperm (spermatozoa) and eggs (oocytes). Physiologically normal eggs and sperm are only available from whole animals. They can only be reliably obtained of a defined age and stage of maturity using the superovulation procedure. The work we plan is either basic research into mechanisms of signalling, or else exploring the effects of compounds on function and viability of normal eggs. There are too many unknown parameters for a computer-based modelling approach.

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

The non-animal alternative that was considered involves the culture of primordial germ cells into gametes.

Why were they not suitable?

The culture of primordial germs cells into fully grown and mature egg or spermatozoa is a very inefficient process. It involves prolonged culture, and this generates many abnormal gametes that do not fertilize or develop normally. Hence the sperm and oocytes/eggs produced are not of the same quality as those derived from animals and of little relevance to human fertility. In addition, the culture of these germ cells generally involves a phase of development when the growing oocytes are surgically transferred into host mice. This means they are often not really ‘animal free’ and will likely involve more animal harm and distress than our current proposals.

Reduction

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

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

During the course of this project, we will conducting many different experiments testing more than 100 different individual hypotheses. We will also design new types of experiments, so it is not possible to estimate sizes of each effect in advance. We measure events in single cells, and the ‘n’ numbers for our statistics are applied to the eggs, and not to the number of animals. Consequently, the number of animals we plan to use is based upon how many eggs we can used per experimental day. The superovulation procedure generates about 20 eggs per mouse or hamster. For superovulation we expect to use an average of 3 mice per experimental day for 200 days per year, for 5 years. Hamsters will primarily be used for about 1 year with superovulation on approximately 100 days with 2 hamster per day.

Mice are also required for maintenance of a colony to generate males that make sperm that are deficient in fertilizing eggs. Our mouse numbers incorporate running a colony of such mice for a period of 3 years during the course of the licence. The colony would consist of at least 4 breeding pairs at any one time and we estimate about 7-8 pups per mouse.

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

We use a standard superovulation protocol that has been previously optimized. We reduce the number of animals by the sharing of eggs collected between research projects. We need to use at least two mice per experimental day to ensure enough material for one project. However, as we add in more projects the number of animals does not increase in a linear manner. Hence three projects can typically be run with four mice per day, so we arrange for the simultaneous use of animals on experimental days. When we breed mice that lack the PLCz1 gene we will generate males that are of a wild type, heterozygous, and homozygous knock out. We will use all of these males for experimental work since the wild type and heterozygous mice will be used as controls for the PLCz1 knockout mice. This will reduce the number of other males that would normally be culled for control fertilization 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 share the use of eggs between projects on each experimental day. We are also involved in projects to produce computer models of the signalling changes in eggs at fertilization. These approaches are a supplement to experimental work because not enough of the relevant parameters are known in eggs.

Refinement

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

Which animal models and methods 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 wild type mice for most studies because we want to study events of normal fertilization in mammals. The CD1 mice are a widely used strain throughout the world and so our data is directly comparable to other research work. Superovulation of these mice is already considered a mild procedure. The genetically modified mice that we will use have a gene deletion for a sperm specific protein called PLCz1. This protein is only found in sperm, and only involved in fertilization. These mice have previously been created and bred in other laboratories and the only known phenotype of the male mice is reduced fertility.

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

Eggs from marine invertebrates (sea urchins), or from other vertebrates (frogs), have been used in many previous studies of fertilization that established some fundamental features of egg activation. However, we now study at least two aspects of fertilization that have characteristics only seen in mammals. First, we are investigating the role of a particular sperm protein called PLCz1 that causes prolonged oscillations in calcium signals in mammalian eggs. The PLCz1 protein is key to mammalian fertilization, but it is not found or in most non- mammalian species. The prolonged calcium signals we study are also only seen in mammalian eggs.

Second, we study changes in mitochondrial metabolism in eggs. The metabolic needs of mouse eggs are very similar to human eggs and embryos. This is evident from the fact that eggs and embryos from either species can be cultured in the same culture medium. This remarkable similarity in metabolic requirements is not seen with non-mammalian embryos which grow in medium without any metabolites (such as sea water), or in eggs that rely on large internal store of energy (e.g. yolk in fish or frog eggs).

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

The animals will be monitored on a daily basis for any harmful effects by the facilities staff and by the research staff who inspect the animals in procedures nearly every day. We have not experienced any harmful effects of superovulation and there are no reported for expected specific harms from the genetically modified mice that we will breed. Nevertheless, we will commit to finding refinements to the superovulation method, such as subcutaneous injections, rather than continuing to use i.p. injections routinely. Any welfare issues of our mice are likely to be the same as those found with standard breeding and maintenance of mice.

What published best practice guidance will you follow to ensure experiments are

conducted in the most refined way?

We shall follow the ASPA guidance and the ARRIVE guidlines on animal monitoring and maintenance.

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

We are informed of developments in 3Rs by regular emails and postings from our NC3Rs Regional Programme Manager.

30. Plasticity of cortical brain function in health and disease

Project duration

5 years 0 months

Project purpose

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

Key words

Plasticity, Neurodegeneration, Behaviour, Neural networks, Mouse

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

Our objectives are:

- to understand how experience changes the activity of brain cells (this is called brain plasticity)
- to establish whether atypical brain plasticity may provide early indicator of brain disorders such as dementias.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 is designed to provide knowledge about how experience changes brain function. We will make measurements from rodents, including mouse strains that have

been genetically modified to model degenerative diseases of the brain.

Learning (often called plasticity) is supported by changes in the activity of nerve cells in the brain, and in the way these cells communicate with each other. Plasticity is also important for the brain to adapt to changes that occur during normal development and ageing, or during disease. However, we do not know why some patterns of brain activity allow plasticity, and some do not. Our measurements are designed to provide knowledge about which patterns of brain activity are important for plasticity.

Dementias, including Alzheimer's Disease, are a major threat to human health - about 850,000 people in the UK already suffer dementia, projected to double in the next 20 years. Our measurements are designed to show how these diseases affect brain plasticity. They are also designed to help develop new biomarkers, because related measures can be made in humans via recordings of brain activity through an electroencephalogram (EEG). Our measurements will help better understand why the EEG is changed in dementia, and how the EEG may be used to detect dementia at early stages, or track recovery during potential treatments.

Neurodegenerative disorders such as dementia have widespread impact on the cerebral cortex but the main target of research has been its impact on memory and cognition, processes that are poorly understood even in healthy humans and disease model organisms. Sensory plasticity relies on the intrinsic cellular and synaptic mechanisms thought important for learning and memory in other brain areas. Measurements of sensory plasticity therefore offer simple, direct measurements of plasticity in brain circuits, and a potential target for understanding, detecting and tracking neurodegeneration.

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

The major output of our study will be new knowledge about how plasticity and degeneration changes the activity of brain cells in rodents. These results will be described in publications in journals, and in presentations both to other scientists, clinical researchers, and the general public.

Objective 1: Our major aim here is to understand what guides long-lasting neuronal plasticity in the adult brain. Previous measurements have focused on understanding changes in the activity of individual brain cells. Our measurements instead focus on how this plasticity is supported by activity in networks of brain cells. Our overall hypothesis is that long-lasting plasticity is guided by particular patterns of activity in the connections between excitatory and inhibitory cells. We expect that our measurements will show what patterns of activity, in which cells, are important for long term plasticity. Our research outputs will therefore provide important information about the factors that can drive faster and stronger learning, which is also likely to have longer-term benefit for understanding how to recover function in people with sensory impairments.

Objective 2: Our major aim here is to understand how degeneration affects network activity and therefore plasticity in the brain. We predict that degeneration has an early impact on communication in brain networks, thereby reducing plasticity and impairing brain function. We expect that our measurements will show how dysfunction of molecules important in degeneration (such as tau and amyloid) changes patterns of activity in the brain, and whether these changes can be used to detect or predict degeneration. Our research outputs will have the short-term benefit of understanding brain activity and behaviour in rodent models of dementia, and will have the longer-term benefit of informing the

development of non-invasive measurements in mice and humans that can better detect or track brain dysfunction.

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

The knowledge that we will generate will primarily be of benefit to other scientists who are interested in understanding plasticity and degeneration in the brain, in humans and in other animals. During the life of this project, the measurements we make will be presented at national and international conferences, and published in academic journals, with open access to the article and data whereever possible.

Some of our measurements are designed to provide information about how degeneration changes the electroencephalogram (EEG), and we hope that these measurements will help in the design of better tests to detect or track degeneration in the brain. At first, these results will have most benefit for other scientists interested in rodent models of brain degeneration. However, we hope that in the longer term that our results will be able to help guide better tests in humans, especially if we find that different types of degeneration have different 'signatures' in the EEG.

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

We make every effort to publish our results in journals that allow open-access, and to provide the raw data in a way that can be used by other scientists. Our experiments are designed to provide knowledge regardless of the outcome of the experiment, and are therefore usually publishable.

Species and numbers of animals expected to be used

- Mice: Approximately 3000 animals

Predicted harms

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

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

Our study will focus on adult rodents, particularly mice.

We have chosen to study rodents, because they are mammals and therefore share strong similarities with humans in the brain pathways that we need to study. In addition, developments in genetic technologies allow the ability to target specific types of cells in the mouse brain, or to introduce genetic mutations that model known correlates of degeneration in humans.

We will focus our study on adult animals because we are primarily interested in the effect of experience and degeneration after the brain has reached a stable state, rather than the changes that occur during development.

We will make measurements from live animals, because our experiments are aimed at understanding patterns of activity in brain networks support plasticity, in normal brains and during degeneration. Our measurements focus on sensory plasticity, and therefore require

intact connections between sensory organs and the brain. The patterns of activity, particularly those dependent on connections between brain areas, are also severely disrupted in brain slices.

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

Most animals will have an operation, performed under general anaesthetic and using aseptic surgical techniques, to allow brain recordings. The skull is exposed, small holes are made to allow access to the brain, and a lightweight device is attached to the skull. In some cases, small electrodes or optical guides (usually less than 5 mm long, and 1 mm wide) are implanted in the same procedure. Everything is secured with dental cement or similar bonds before closing the wound. During these procedures we do not anticipate anything more than some minor bleeding which will be stopped immediately during the operation. The animals recover from the anaesthetic in a thermally controlled box, and are then returned to their home cage, where they remain under close observation. The animals may experience some post-operative pain, which will be controlled by the use of pain-killing agents. The severity level of these surgeries is moderate.

After recovery from surgery, the animals are gradually habituated to handling, and head-restraint where it is needed. Animals may then be trained to obtain a food or liquid reward for doing a task.

Measurements are made while a sensory stimulus (e.g. a pattern of light) is presented, or while the animal is exploring a small environment. Typically, these measurements take 2-3 hours to complete. In some animals, these measurements will be made once per day over the course of about a week. In other animals we will instead make less frequent measurements (for example, every 2 weeks) over the course of several months, to track long term changes in the brain. All animals will be humanely killed when these measurements are concluded. The severity level of these procedures will be mild or moderate.

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

In some animals, a genetic modification to reproduce a potential cause of brain disorder will be introduced. Some of these lines of animals show little evidence of potentially harmful phenotypes at any age, but others show potential for harmful phenotypes at older ages, and we will therefore conduct our experiments before these ages are reached. The severity level of breeding and maintenance of these animals will therefore be mild or moderate.

Most of the expected adverse effects relate to the surgical implants, and problems derived from the surgeries, such as anaesthetic death, post-operative pain, post-operative infection and occasionally, as a result of infection, post-op implant failure. These potential effects will be mitigated by careful anaesthetic and aseptic surgical techniques, careful monitoring daily in the post-surgical period and prompt treatment of any infections that might develop. We are also constantly working to develop light- weight devices to measure neural activity, which have three main advantages: reduction of the adverse effects, increase of the amount of data we can obtain from each animal (i.e. reduction of the number of animals), and, most importantly, reduction of discomfort for the animals

The procedures we will use are generally well tolerated by the animals, and do not impede

their natural or trained behaviours or their health. We expect some signs of anxiety when animals are first exposed to handling or restraint, and this will be controlled by gradual habituation to each procedure. In addition, animals participating in tasks may need food or water restriction (never both) for motivation – their food and water intake will be monitored carefully alongside their weight, but these animals may experience moderate weight loss. If they show signs of ill health or more substantial weight loss, they will be taken off the task and given free access to food and water, and experiments will only be recommenced if they recover well. If they cannot learn the task they will be removed from the study and 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)?

Mild: some (ca. 10%) of the animals will only undergo simple behavioural testing.

Moderate: most (ca. 80%) of the animals will go through a moderate surgical procedure. In these animals, behavioural or neural measurements are usually made in awake state after recovery from surgery.

Severe: none of the animals are expected to go through a severe procedure.

Some animals (ca. 5-10%) may go through terminal, non-recovery procedures to make physiological measurements under anaesthesia.

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

- Killed
- Used in other projects

Replacement

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

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

The questions we are investigating require measurements from individual nerve cells in the brain, which are only possible in very limited circumstances in humans (usually during surgery for epilepsy). These measurements also require the presence of normal sensory inputs and intact brain pathways connecting the nerve cells. These pathways have not yet been replicated in vitro or in silico, and answering these questions therefore makes it necessary to perform experiments on intact animals.

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

We already use alternatives such as human measurements and computational modelling where possible. For the basic questions around plasticity we have considered making electroencephalogram (EEG) or functional MRI measurements from humans. We have also considered making EEG or fMRI measurements from humans with specific mutations in Tau, Amyloid or other proteins. Indeed, our long term aim is to be able to use the

knowledge about EEG and related measurements, obtained from animals with very specific mutations in these proteins, to help provide precision tests for the detection and diagnosis, or tracking of treatment effects in humans. However, it is currently not feasible to conduct these measurements in appropriate humans in the controlled and longitudinal way we need to develop these measurements towards clinical implementation.

Why were they not suitable?

EEG and fMRI measurements are indirect measures of local neural activity and would therefore not allow us to make the inferences that we need to be able to make. Further, it is not yet possible to measure from substantial groups at early and late stages of disease. We believe that our measurements will put us in much better position to be able to make predictions for these measurements.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 we will use on the basis of statistical advice and previous publications from our own group and others. Where previous measurements of variability are available, and power calculations are therefore possible, we have made power calculations assuming two-sided tests with power 0.8 and alpha 0.05. Where previous measurements are not available, we have used the resource equation.

The number of animals required is relatively low because we often make large-scale recordings from individual animals. For example, recent advances in technology mean that it possible to record electrophysiological or imaging signals from 200-600 neurons in a single session. As the activity of neurons within the same animal are not independent from each other, however, we will usually use linear mixed-effects models (LMMs) to account for these dependencies when pooling the data from all recordings. LLMs require at least 5 groups, i.e. animals, for a robust estimate of variance across groups. Where we are making measurements of longer-term plasticity or field potentials, we cannot take the same approach. The resource equation and our experience establishes that cohort sizes of about 10 animals provides a generally appropriate number in these cases, at least for planning purposes, where we are making single comparisons (e.g. stimulus-dependence, or strain- dependence).

For the measurements in Objective 1.1, we think it likely that we will need to characterise activity in 5- 10 brain areas (visual cortex, auditory cortex, and their thalamus and mid-brain connections), and will make more detailed measurements from 1 or 2 areas in transgenic mice to characterise different cell classes. Therefore, we expect to need to make good recordings from about 50-75 mice, and believe it is likely we will need to make pilot measurements. We estimate that about 75% success rate in targeting each area, 70% success rate in training, and 95% success rate in surgeries, so therefore expect to need 100-150 mice entering the protocols to produce the relevant recordings. For the measurements in Objective 1.2, we think we will need to characterise activity in 5-10 brain

areas, thus 50-100 mice, and thus 100-200 mice entering the experimental protocols. Thus 200-350 mice will be needed for Objective 1.

Our ability to estimate the number of mice required in Objective 2 is poorer, because we do not yet know how many timepoints we may need to consider in each transgenic strain of brain disease. At a minimum, if we study 3 transgenic strains and their relevant controls, at one time point, then we expect to require 60 animals to measure short term plasticity, and another 60 to measure long term plasticity. In addition, we expect to need to study the connectivity profile (neural tracing) of about 10 mice in each group, with similar conversion rates. Thus about 360 mice entering the experimental protocols for Objective 2.

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

Experiments are designed according to the NC3Rs ARRIVE Guidelines to ensure experimental rigour (blinding, randomisation etc) when possible.

Whenever possible, the animals will be tested for periods of several weeks -as long as the animal is healthy- and thus considerable information is obtained from each animal, minimising the total number used.

Suitable statistical analysis (e.g. ANOVA) and/or the use of linear mixed-effects models will minimise the number of animals needed to overcome inter-subject variability, and more reliably test the statistically significance of an observed effect. When necessary, we will seek for statistical advice from colleagues and collaborators.

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

We do not maintain lines on the vague hope of using them at some point - we obtain breeders when we know that they are needed for the questions posed, and reduce them when those experiments are complete.

We usually conduct pilot experiments to make sure that the choice of sensory stimulus that we use is appropriate, and where possible to estimate the variance in the data that we will be collecting. These pilot experiments can often be included at the end of experiments designed to address a previous question, which means that additional animals often do not need to be used. We can therefore adjust our power calculations and related estimates as the experiments proceed, and iteratively reduce the number of animals required to address the questions posed. In addition, we make use of available data to answer questions where that is possible. For example, we have previously conducted secondary analysis on data from the Allen Brain Institute and published the results of these investigations.

Similarly, where possible we take advantage of such datasets to provide pilot data through which to develop analyses and design experimental paradigms.

The sensory cortices are the best understood of the cortical areas, and there are now several models that start to provide reasonable predictions for the activity of brain cells in these areas. We use these models routinely (e.g. models of receptive fields in visual cortex) to make predictions about the responses of the nerve cells that we will measure, however most of our questions address the pattern of activity across nerve cells, and the

impact of experience on these patterns. The large-scale, dynamic aspects of nerve cell activity are not yet well captured by models, but we believe that our and other data will improve this situation over the next few years.

We plan to use both male and female animals for all the experiments, unless relevant phenotypes are sex-linked.

Refinement

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

Which animal models and methods 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 experiments will be conducted on rodents, because the relevant brain networks are well characterised in this species. Because rodents are, like humans, also mammals, we know a good deal about how we can make inferences about human brain networks from the experimental measurements we make. In addition, genetic technologies enable the generation of mouse models of brain diseases (such as Alzheimer's Disease), that can recapitulate key aspects of these disorders in humans. Most of our experiments involve performing surgery under anaesthesia to implant recording devices in the brain.

Our measurements are then made in the awake state when the animal has fully recovered - the process of making measurements from the brain does not cause pain, suffering or distress to the animals.

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

The measurements we propose to make address the organisation and function of brain circuits, in normal brains and during brain disorders. Many of these functions are 'integrative', that is they involve the concerted action of many nerve cells in many parts of the brain. It is important, to make inferences about healthy and unhealthy human brains, that we make measurements in species where these circuits are similarly organised. We cannot make these measurements in flies because these integrative nerve circuits in flies are very different to those in mammals such as humans.

The measurements we propose to make are aimed at understanding plasticity and disorders in the adult brain, when the circuits between nerve cells have already been largely formed. The brain is still developing rapidly at earlier (e.g. neonatal) stages of development, and this makes interpreting the measurements very difficult. We will make measurements from awake animals because (1) the measurements we propose to make require comparison of activity in the brain before and after exposure to a stimulus (learning), and the timescales of learning extend over days or weeks; (2) anaesthetics have profound effects on brain function, especially in small animals such as mice, and it is very difficult to know how to interpret measurements of brain function in anaesthetised state.

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

Post-operative care and pain management: We will minimise pain and discomfort during and after all the procedures performed with animals. Surgeries will be performed under general anaesthesia.

Analgesics and antibiotics will be administered as needed during and after surgery to minimise pain and reduce the risk of infection, respectively. The surgeries will be performed by competent staff to minimise side effects and the length of the surgery. All animals will receive post-operative care to ensure a normal recovery. We closely monitor animals in a separate, heated area, until they are recovered from anaesthesia and are moving freely. We have designed our experimental regimes so that we can avoid surgery on Fridays, and we closely monitor the animals for at least 3 days after surgery, during which time they are also provided with oral pain relief medication.

Handling and training of animals: We have refined our procedures by placing strong importance on training research staff in better handling, and dedicating several days to initial familiarisation of animals to research staff before surgical or experimental intervention. We have refined our procedures so that instead of holding by the tail we now routinely use 'hand cupping' or where possible 'cardboard tunnels' to move animals from holding cages to transport boxes and apparatus. Where animals need to be temporarily restrained during the course of measurements, we have refined our procedures so that animals are gradually habituated to the apparatus and the restraint (usually over 5 days). Behavioural testing: External stimuli that could result in stress for the animals (e.g. bright light, loud sounds, etc.) can be minimised by isolating the behavioural apparatus. If an animal shows signs of stress during the behavioural task, it will be removed from the task until calmed, or completely removed and euthanised if it fails to recover. Fear responses to the behavioural apparatus will be minimised by providing animals time to habituate to the apparatus at the start of the experiment, as above.

Food or water restriction will be used only when strictly necessary to enhance animals' learning and motivation. Restrictions will be applied to only a minimal degree so that rewards presented during the task are motivating. We will be vigilant of new published methods for positive reward, in search of a suitable replacement. In a subset of animals, we will conduct automated behavioural training within their home cage, allowing the animals to train when they want to during the day or night, without leaving their cage and avoiding any contact with the experimenter. This approach maximises the rate of learning while minimising stress in the animal. Where head restraint is required to avoid animal movement, distress will be minimised by gradual adaptation.

Environmental enrichment: In the last 12 months we have refined standard cage design so to provide greatly increased space between the floor and the ceiling, substantially reducing the possibility that implanted animals become entangled. This advance means we can now also routinely enclose running wheels in the mouse cages without entanglement.

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

We are following published guidance including:

Experimental design: https://arriveguidelines.org/arrive-guidelines Experimental

procedures:

Barkus et al. (2022) Refinements to rodent head fixation and fluid/food control for neuroscience. Journal of Neuroscience Methods, 381: 109705. https://doi.org/10.1016/j.jneumeth.2022.109705

Prescott MJ, Lidster K (2017) Improving quality of science through better animal welfare: the NC3Rs strategy. Lab Animal 46(4):152-156. doi:10.1038/laban.1217

Turner, Brabb, Pekow, Vasbinder (2011) Administration of Substances to Laboratory Animals: Routes of Administration and Factors to Consider. J Am Assoc Lab Anim Sci. 50(5): 600–613

Wilkinson MJA, Selman C ... Flynn JN (2020) Progressing the care, husbandry and management of ageing mice used in scientific studies. Laboratory animals, 54(3):225-238. doi: 10.1177/0023677219865291

Breeding / colony management: https://nc3rs.org.uk/breeding-and-colony-management, and https://nc3rs.org.uk/3rs-resources/breeding-and-colony-management/worked-example-intermittent- breeding

Guiding Principles for Behavioural Laboratory Animal Science: https://www.lasa.co.uk/wp-content/uploads/2018/05/LASA_BAP_BNA_ESSWAP_GP_Behavioural_LAS_Nov13.pdf

Animal monitoring: https://nc3rs.org.uk/3rs-resources/grimace-scales NC3rs research gateway: https://f1000research.com/nc3rs
How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We have already made active contact with our local NC3Rs programme manager to discuss the proposed experimental design. We will ensure that research staff and project licence holders regularly attend 3R's workshops, including those run by the NC3Rs team.

The PPL holder will attend at least one online 3R seminar each year, to keep up to date with new advances in the 3Rs (https://nc3rs.org.uk/events)

Receiving regular newsletter updates from N3CR on advances in 3R practices, and regular review of the research gateway (https://f1000research.com/nc3rs)

The PPL holder will have regular discussions with our NACWO and NVS, on new ways to refine our methods, according to new advances in the 3Rs (e.g. newsletter, seminars)

31. Study of the melanocyte lineage in development, tissue maintenance and disease

Project duration

5 years 0 months

Project purpose

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

Key words

Melanoma, Pigmentation, Development, Homoeostasis, Disease

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim of this project is to understand the embryonic development, function/maintenance/homeostasis and disease states of melanocytes - the cells in our skin and hair that produce pigment.

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

Why is it important to undertake this work?

Mouse melanocytes represent an excellent model to understand the basic developmental processes required to generate healthy organisms. Failure of these processes in melanocytes leads to a number of classical developmental diseases, such as piebaldism (depigmented patches on the chest and forehead) and premature hair greying, that phenocopy the human diseases and have relatively few welfare consequences for the

animals. As such, mouse melanocytes represent a tractable experimental model for human disease. The cancer of melanocytes is melanoma, a disease with dramatically rising incidence in human populations and very poor prognosis if not detected early.

Mouse is a highly tractable model in which to study the genes and genetic variants that drive melanoma experimentally because of the availability and ease of generation of precise genetic models.

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

We will generate data and new knowledge that explain the embryonic origins of the melanocyte lineage their function/maintenance/homoeostasis in adult tissues and their disease states including in metastatic melanoma. In doing so we will: publish peer reviewed research articles in high impact academic journals; win follow up funding from UKRI and charity based funding streams; generate new transgenic models to investigate the melanocyte lineage and tissue homoeostasis to benefit the pigmentation, developmental biology and cancer fields; identify potential drugable targets in pathways implicated in melanomagenesis and pursue commercial patents where applicable. Melanoma outcomes are good if found early. However metastatic melanoma is currently incurable as despite initial good response to immunotherapies, relapse is almost inevitable due to the emergence of resistant melanoma sublineages.

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

We will mainly produce primary research publications and review articles, present our data at local and international conferences and generate datasets to deposit for further research.

In the short term (5+ years) this project will develop models to examine melanocyte embryonic development and the function and the maintenance of melanocytes in the skin. It will establish how these behaviours are altered in disease states and in the initiation and development of melanoma. These models will include new transgenic methods to label melanocytes or the surrounding tissue with genetically encoded sensors (for example labelling cell cycle progression or lineage tracing probes.

These will be shared with other developmental biologists and biomedical scientists. The work that we undertake will also establish the key assays required to develop culture and live imaging of adult mouse skin as a preclinical model for melanoma initiation and development - we will prioritise publishing the method as soon as possible.

In the medium term (4-8+ years), we will collaborate with immunologists, engineers and chemists to exploit our skin culture models. Future grant applications will: explore the early interactions of melanoma initiating cells with their environment (including resident dendritic cells and the circulating immune system) so that we can develop new diagnostic strategies and drug screening platforms; develop 3D printing in skin for diagnostic and therapeutic applications. Therefore this project will greatly benefit diagnostic/treatment strategies of human disease as well as informing the study of basic melanocyte (and skin) research.

In the long term (5+ years) our outputs will be used locally, nationally and internationally to stimulate new directions for research into melanoma metastasis, treatment and therapy or into the basic biology of melanocyte homoeostasis. Our new mouse models (funded by NC3Rs) will reduce animal usage by replacing the use of thymidine analogues and

generating more data from fewer experimental crosses. They will be applicable to other body systems and so will benefit the entire community of developmental biologists and biomedical scientists - generating worldwide 3Rs impact. Our skin culture model will be applied by ourselves and shared with researchers at other establishments to investigate many aspects of melanoma biology and the response of melanoma cells to therapy in vivo. Because our model incorporates live imaging it has clear advantages over the existing models both scientifically and from a 3Rs perspective.

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

We will maximise our outputs through multidisciplinary collaboration, open access publication and sharing of datasets. New knowledge will be disseminated in exclusively open access peer reviewed journals. Where datasets are generated they will be shared using open data standards using public repositories and creative commons licensing where possible. New publications, tools and software 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 e.g. F1000Research.

Species and numbers of animals expected to be used

- Mice: 11000

Predicted harms

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

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

We will study embryonic and adult mouse skin. Ideally we would study human skin but at present the methods are not sufficiently advanced to address the key questions - especially during embryonic development. Therefore we have developed an ex vivo, organ culture system for mouse embryonic and tail skin which provides the most realistic model currently available to study different cell types in living skin. The mouse tail is a good model of human skin because unlike other regions of the mouse body it contains dermal and epidermal melanocytes. Furthermore, we are able to take advantage of mouse genetics to develop highly tractable models.

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

We will use genetically modified ‘transgenic’ mouse lines to study specific cells types, such as melanocytes, the cells responsible for producing the pigment in our skin. These transgenic lines have three components: a 'driver' strain that produces an enzyme called Cre-recombinase in the target cell type, a 'reporter' strain that produces a fluorescent protein when activated by Cre, and a 'floxed' gene of interest that can be turned on or off by Cre.

By combining these components, we can specifically label the cell type we want to study and introduce a genetic change whose effects we can observe. Cre is expressed in a cell types specific manner but if we require temporal control we can use CreERT2. In order to activate CreERT2, we will use a substance called tamoxifen, which can be administered

through injection, gavage (oral administration), or topical application, depending on the experimental stage (adult or embryonic).

For adult experiments, after activating the system with tamoxifen (if required), we will euthanize the animals so that we can study the skin cells in a laboratory setting. For embryonic experiments, pregnant animals will be euthanized, and the embryos will be removed for further study. If the embryos are past a certain stage of development (E14.5), they will also be euthanized before study.

To study melanoma (the cancer of melanocytes) the genetic changes required to initiate the disease will be induced using the above system. The animals will be closely monitored, and they will be euthanized once the disease has reached a specific clinical score defined in the experimental protocol (typically about 3 months after induction). Sometimes tumour cells will be introduced to normal animals to under stand how they metastasize to distant sites.

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

The majority of studies will focus on melanoblast behaviour in embryonic or adult skin culture and no adverse effects are expected for adult mice, dam or offspring. However the majority of animals will experience mild severity as they will be ear clipped for genotyping purposes. Studies that investigate melanomagenesis will be carefully controlled for severity. In these studies animals may develop skin tumours and in some cases may show metastasis to the lymph nodes, liver, lungs or brain. Animals will be monitored for tumour growth and metastases regularly. If tumours become ulcerated or if the largest tumour reaches 1.5cm 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)?

5-10% of mice may experience a moderate severity.

90-95% of mice will experience mild severity as they will be ear clipped for genotyping purposes.

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?

This project will use embryonic and adult mouse skin as a model for human skin to

investigate the melanocyte lineage in development, tissue maintenance and disease. Much research has been conducted in cell culture based models to address these questions. Unfortunately this research is flawed because the cell culture environment is too far removed from the in vivo environment and the cell lines used are transformed. Furthermore, the key lineage relationships between melanocyte populations and the homoeostatic mechanisms that maintain them have not been defined and can only be explored in an animal model. The use of mice is therefore pivotal to the application and the experiments proposed would not be tractable in another experimental system. Furthermore because it is easily accessible mouse tail has huge potential for the development of preclinical non-invasive imaging techniques in this field.

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

An alternative approach would be to use non-invasive imaging of human skin. We are considering methods to develop label free imaging and are exploring Raman spectroscopy on melanocyte cell lines to see whether we can develop label free methods for the future. However at this point there are no alternatives to address our experimental questions.

Why were they not suitable?

It is not currently possible to delineate the individual cell types of the skin label free in human and there is no way to study live embryonic populations in human.

Reduction

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

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

We have used power calculations, experience from previous studies and the NC3Rs experimental design assistant to determine the number of experimental animals required. Our development of mouse reporter lines that express multiple markers has already made a significant contribution to reducing animal usage becasue they require the crossing of less mouse lines to generate our experimental animals.

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 PREPARE guidelines to reduce waste, promote animal alternatives, and increase the reproducibility of our research. All the mouse lines in this

study will be imported from other establishments. We will assess breeding performance and ensure the minimum numbers of animals are used for breeding and maintenance.

Refinement

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

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

We will use genetically modified mouse models. To induce gene expression in animals or to specifically label melanocytes, some animals will be given substances by mouth, injection, or through food. Oral gavage or injection can be necessary to induce a rapid change in gene expression. We are predominantly interested in the early events of tumorigenesis and will prioritise studies in ex vivo embryonic and adult skin culture. In some cases it may be necessary to age animals with skin tumour in order to track progression on different genetic backgrounds. This is the only method that allows monitoring of the in vivo consequences of these genetic changes. We minimise the suffering caused to the experimental animals by identifying unexpected phenotypes as quickly as possible.

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

Ideally we would study human skin but at present the methods are not sufficiently advanced to address the key questions. I have developed an ex vivo, organ culture system for mouse tail skin which provides the most realistic model currently available to study different cell types in living skin. Mice are used because they are the lowest sentient species that have melanocyte populations in the skin equivalent to the human populations. Furthermore, use of genetically modified mice allows novel experimental investigations, which are not yet possible with other species.

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

The main refinement possible is in the monitoring of emergent phenotypes from our animal crosses. Where a phenotype (e.g. melanoma secondary metastasis) is expected we will monitor animals daily until we understand the penetrance of any phenotype in said cross. We will keep the monitoring frequencey under review after this inititial period.

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?

I have been in receipt of NC3Rs funding since 2013 including two PhD studentships and a pilot grant. I am currently on the NC3Rs studentship assessment panel. I therefore have a good oversight of current developments in the field through NC3Rs newsletters and through reviewing grant applications. We are actively developing biosensor expressing muse lines and imaging techniques that result in fewer animals being required for genetic crosses and the generation of larger data volumes from a smaller number of experiments.

32. Subclinical disease resistance and resilience in ruminants

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

Parasites, Nematodes, Nutrition, Plants, Extracts

Animal types	Life stages
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?

The overall aim of this work is to study the impact of nutrition on resistance and resilience in sheep, during subclinical disease. As subclinical disease is defined a slow reduction in feed intake (or feed efficiency and lack of weight gain) that is not easy to be identified by farmers. The first objective is the establishment of a subclinical parasitic infection under controlled experimental conditions. The second objective is to investigate whether novel plants, such as heather or extracts rich in secondary plant metabolites, such as bark extracts, reduce the impact of parasitism on resilience (as measured by performance) and resistance (as measured by immune response). The third objective is to investigate whether parasitised sheep can select a diet that is rich in plant secondary metabolites (such as heather) to self-medicate.

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

Why is it important to undertake this work?

Subclinical parasitic disease is responsible for suboptimal health and welfare and production losses in livestock and sheep in particular. It is also known to play a role in the development of drug resistance, which may result in overuse of drugs. This is because in subclinical disease the symptoms are not evident (e.g. reduced feed intake or slow growth), which may result in misdiagnosis of the disease, which is one of the main factors of drug overuse and thus development of resistance. Alternative control of sub-clinical disease remains paramount to improve production revenue and animal welfare and reduce the rate of resistance development to anti-parasitic drugs. Reduced reliance on drugs also alleviates public concerns on drug residues in animal products and the environment and assists development of organic systems of animal production.

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

At the end of the project, expected outputs include peer review publications, presentations to scientific audiences and stakeholders. In this programme of work, the main focus is on in vitro screening of plant extracts originating from UK sources and in vivo screening of crude plant extracts (i.e. a collection of many compounds). We are hoping that by the end of this programme we will have identified purified compounds from bark and/or heather extracts that can potentially be tested in vivo in sheep. We are expecting that this work will have an impact on animal welfare, as it will result in a reduction in the use of anthelmintics without penalising the health of the animals infected with gastrointestinal parasites.

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

In the longer term, once the outputs are published, other researchers with interest in these areas, as well as stakeholders such as animal feed industry and livestock co-operatives will benefit from these developments. Animal health and welfare, as well as the environment will benefit from the work, as parasite control will be achieved with minimum anthelmintic input.

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

This project is based on collaborative work between leading academics and industry. It is interdisciplinary work between parasitology, ecology, forestry and chemistry. Knowledge will be disseminated to diverse audiences and work will be submitted for publication in open access journals to maximise impact. Resources will be made available to fellow researchers via open data sharing, as required by UKRI and other funding schemes.

Species and numbers of animals expected to be used

- Sheep: 400

Predicted harms

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

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

Sheep are the natural hosts of these parasites and growing sheep are particularly susceptible as they haven't developed immunity to them yet. So these are the life stages of hosts we will be using.

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

Typically, sheep will be infected with parasite larvae at a level where a subclinical infection will be established. During the infection, donor animals will be fitted with a harness to collect parasite eggs in faeces, which will be incubated to hatch and develop into infective larvae which will then be used to infect more sheep. Infected sheep may be offered different levels of plant extracts or will have different levels of heather offered to them. Sheep will be faecal sampled throughout the experiments to determine faecal egg counts in their faeces. They may also be blood sampled at key time points during the infection; they may experience mild discomfort during blood sampling without lasting harm. Animals may undergo changes in their diet which are not expected to cause distress, but some diets result in reduction in feed intake and weight gain, due to low palatability.

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

Sheep will be orally infected with nematode infective larvae at a level to establish a subclinical infection. They are not expected to experience major harm from the oral administration of the parasites. Based on our previous experience, a 20-25% reduction of food intake is expected in sub-clinically parasitized animals. No severe adverse effects of PSM (Plant Secondary Metabolites) exposure are expected.

Previous evidence has shown that intake of crude PSM extracts above 16% of estimated dry matter intake may have detrimental effects on sheep and will be avoided. Heather feeding can reduce intake if offered at around 20% of the dry matter intake initially, but this effect is short-lived (previous evidence showed that animals adapt after a few weeks).

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

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

The expected severities in sheep may include sub-threshold (25%), mild (50%) and moderate (25%). We are proposing moderate severity for 25% of the animals as these animals will be infected, routinely faecal and blood sampled and may be given plant compounds or access to heather grazing. They may also show a reduction in feed intake and or growth.

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?

This program of work aims to assess the impact of a host's nutritional environment towards sub-clinical livestock disease and in particular gastrointestinal parasitism. Disease affects the host animal in a range of ways, including through its nutrient ingestion, digestion, production and behaviour. It can therefore be anticipated that interventions affecting (the outcome of) sub-clinical disease do so through the involvement of multiple mechanisms of the host's physiology, including its immunology, endocrinology, digestive physiology and neurology. Mathematical methodologies to study some aspects of sub-clinical disease have been developed to test in silico a range of management scenarios that could impact (sub-clinical) disease. In addition, a small number of sheep are needed as helminth donors, which cannot be produced in vitro. In vitro methodologies, such as egg hatch and larval motility assays, will be used as much as possible to inform animal studies. In particular they will be used to screen a large number of plant extracts and the most active extracts will then be used in animal experiments. Additional in vitro studies, such as cytotoxicity tests, will be used to assess toxicity of the extracts prior to their use on animals, to reduce the possibility of side effects.

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

Large scale in vitro screening to reduce the number of extracts tested in vivo have been considered and will be implemented as part of this project. In vitro assays such as egg hatching and larval motility tests will be used prior to any in vivo experiments. Donor sheep are required to generate the parasite life stages for in vitro testing.

Mathematical methodologies to study some aspects of sub-clinical disease have been developed to test in silico a range of management scenarios that could impact (sub-clinical) disease.

Why were they not suitable?

The involvement of such a large range of host bodily functions in their response to sub-clinical disease reduces the possibilities to use non-animal experimentation, and thus justifies the use of animals.

Reduction

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

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

Based on previous experiments where the typical variation in the experimental variables was described we were able to calculate, with the help of a statistician 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.

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

To reduce the number of animals used in the project, we need to reduce the variation in the population of the hosts and standardise the conditions under which the experiments are performed as much as possible. To that effect, experiments will be planned well in advance, and animals with similar age and body weight (as much as possible) will be used for the individual experiments. The number of replicates required will be informed through a combination of experience and power calculations. For example, our experience is that variation in performance measures like weight gain is usually higher in parasitized animals than in sham infected animals, and we therefore often allow for less replicates in the sham-infected animals. Resulting a priori unbalanced data sets can readily be analysed through statistical methods like REML. The program of work on anti-parasitic plant extracts will test only those that show strong in vitro activity and possibly in vivo activity from preceding rodent studies. This significantly reduces the number of target animals needed to study plant extract impacts on parasite establishment. Power calculations will be used when expected or desired effect size is known. Where possible, this will be derived from our earlier studies and from the literature. Each individual animal experiment under this project requires approval from Animal Welfare and Ethical Review Body (AWERB). This includes assessment of appropriate number of replicates and use of power analysis. To this effect, a statistician sits on the AWERB, who is also consulted prior to experimental design submission.

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

Apart from good experimental design, the use of in vitro methodologies, such as egg hatch assays and motility assays will be used to optimise the number of sheep used for the testing of the anthelmintic properties of extracts.

Refinement

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

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

The overall aim of this programme of work is to increase our understanding on how the nutritional environment of the host impacts on subclinical disease in farm animals. For this reason, we use farm animals as models but also as target animals, although where possible and appropriate, the underlying hypotheses to be tested and the associated experimental designs will be informed by in vitro work and work on rodents. In addition, sheep are most appropriate to address interactions between host traits and nutrition on gastrointestinal parasitism under grazing conditions. For our sheep studies, we focus on gastrointestinal nematode infection models as our host/parasite system, using T. circumcincta and Trichostrongylus spp. However, other infection models may be explored as and when required for pursuing our hypotheses. These species are the ones in which

response to the pathogen and the course of infection have been well characterised in our previous sheep studies.

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

As mentioned earlier, we will use in vitro screening assays and in vivo testing in rodent animals to reduce the number of sheep used for this programme of work. Subclinical parasitism is not expected to cause any pain or suffering in animals, although it is expected to have an impact of feed intake and performance. As sheep is the organism of target, it is important to validate the in vitro and in vivo (mouse) findings in sheep prior to the implementation of any novel approaches for parasite control.

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

All animals, from the minute they are parasitised, be monitored by trained staff daily (e.g. NACWO, project manager or Named Vet). We build on our previous studies that have confirmed and refined the infection levels required to induce sub-clinical rather than clinical disease. To minimise the possibility of accidentally overdosing the animals, we have introduced an extra baermanisation step to distinguish between dead and alive infective larvae. Infective larvae often appear to be dead (and thus are not counted in the infective larvae dose), which increases the risk of overdosing, but this extra step mitigates this risk.

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

The PREPARE guidelines will be followed.

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

To stay informed about advances in the 3Rs and effectively implement them in our work, we will regularly be checking information on the NC3Rs website and attend Regional 3Rs symposia organised by relevant organisations. Advice from AWERB and the Named Vet will be sought regularly.

33. Production and cryopreservation of GA animals as a service and associated support

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

centralised service, rederivation, cryopreservation, generation

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

To provide a service for generation and cryopreservation of Genetically Altered (GA) mice and Rats at a high and consistent standard to the scientific community .

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

Why is it important to undertake this work?

Genetically altered animals (GAA), particularly rodents are used extensively in biological and translational research. They are used in basic research to elucidate the function of

genes and pathways in a wide variety of biological processes; as models of human diseases to determine pathogenesis and test therapeutic strategies; and to generate therapeutic substances. The human, mouse and rat genomes have been sequenced , however the function of many genes is not known or fully understood. It is not known how the genes interact with each other or how their dysfunction can lead to disease, this is where the use of genetically altered animals will be required to help understand and study disease in detail. Once the genetically altered rodents have been established their sperm or embryos can be cryopreserved to make available for the scientific community both within the UK and Worldwide. Cryopreservation will allow users to put their projects on hold, avoid the generation of unnecessary animals through breeding, but more importantly will prevent the risks of genetic drift when animals are inbred for a long period of time and therefore ensure that the genetic modification is safely preserved. The rederivation of imported or exported lines will be ensured using germplasm compared to using live animals in order to always guarantee the highest health status and comply to the 3Rs.

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

To have generated, rederived , bred and cryopreserved novel mutated strains for use by the scientific community leading to new discoveries and published data.

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

The scientific researchers will benefit from the generation or importation of novel genetically altered (GA) rodent lines, allowing them to investigate many genes in further detail, to publish data and make findings available to the scientific community and general public. A centralised database of cryopreserved lines, sperm and/or embryos will also be available for the scientific community.

Cryopreservation will prevent the risks of genetic drift ensuring that the genetic modification is safely preserved. Therefore, projects can be put on hold if any issues were to present themselves, avoiding the generation of unnecessary animals through breeding and could be restarted at a later stage via rederivation with the certainty of getting the mutation back safely.

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

All members of the service are highly skilled and have been undertaking the procedures on this licence and others for over 15 years . They are all members of the international transgenic list where collaborations and knowledge are shared and questions posted to constantly improve techniques to enable successful experimental outcomes. They are also members of the transgenic community which holds regular training courses and showcases new technologies for the use in Genetically Altered Animal (GAA) production. All animals and lines either produced or rederived at their establishment are placed on the service database, this enables other members or the scientific community access to information on animals that have already been produced or obtained therefore limiting the need for repetition of experiments. The facility also answers regular emails from the UK mouse locator and has transferred many cryopreserved lines that we hold to other members of the scientific community.

Species and numbers of animals expected to be used

- Mice: 6100

- 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.

Mice and rats are the two main type of animals used in fundamental research as their respective genomes have been effectively mapped. The sequences being extremely similar to the human sequence, mice and rats can be used for basic as well as translational research. Users who require our service will be conducting fundamental research, justifying the use of these two types of animals. Rat genome being even more similar to humans, their use can help understanding the role and functionality of certain genes through fundamental basic research and possibly transcribe some data from animals to humans through translational research and move a step closer in order to understand the functions of particular human genes.

In most cases, our work will be carried out at embryo stage between single cell stage (0.5 days post coitum) and blastocyst stage (3.5 days post coitum) according to the procedure being undertaken.

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

All procedures involving the generation of GA animals (IVF, rederivation, embryo injections, electroporations) will require potentially genetically altered embryos to be transferred into pseudopregnant females. To induce pseudopregnancy, females will be mated with vasectomised males before undergoing embryo transfer.

Some procedures covered by this license such as embryo injections for the generation of novel models and IVF will require the facility to order females in as embryo donors. These females will be injected hormones for them to superovulate. Superovulation increases the number of embryos developed by female embryo donors. It will require the administration of hormones intraperitoneally twice with 46-48h in between each injection, causing very little pain or distress to the animals. After the second dose of hormones, in some cases, females will be mated with stud males (IVF does not require natural fertilization as fertilization is done In Vitro, therefore does not require natural mating). After 24h, the females will be separated and humanely killed for embryo collection. Once the embryo procedures have been carried out, they are ready to be implanted into the pseudopregnant females, surgical procedure can occur.

At the time of the surgical procedure, the pseudopregnant female is placed in an induction chamber where a mix of oxygen and anesthetic gas, is distilled in the chamber to provoke loss of consciousness of the animal. Once the animal is anesthetised, it is taken out of the chamber and placed on its stomach on a platform, its nose in a facemask where a mix of oxygen and gas (at a lower % than during induction) is distributed through the mask to maintain loss of consciousness. Pedal reflex (pinch of a toe) is tested regularly to make sure the animal is not experiencing any pain while under anesthesia. The animal is then being administered an anti inflammatory drug as well as a painkiller for analgesia, subcutaneously, causing no pain or distress. We shave the lower back and flank of the animal and sterilise the area. Animals are drapped and an incision on the lower back/flank

of the animal is made, revealing the muscle wall. We visualise the ovary through the muscle wall and make an incision in the body wall at this point. We pull out the fat pad that is attached to the ovary and oviduct revealing the infindibulum and/or uterus. The embryos will then be inserted using a mouth pipette previously loaded with the manipulated embryos. The fat pad-ovary/oviduct are re-inserted into the body cavity and a small stitch will be made in the body wall and the incision on the back will be closed. The surgical procedure will have lasted less than 15 minutes. Animals will be monitored until full recovery of movement is regained. Even though the severity is moderate, adverse effects are unlikely to occur from this procedure. It is a procedure performed by experienced staff previously assessed by a veterinarian. In the rare case an animal is found in pain distress or lasting harm, it will be assessed by a NACWO or a vet and if required, will be humanely killed. 19-21 days after surgery, females are expected to litter down. 21 days after birth the offspring will be weaned and ear sampled and transferred to the end user/investigator's PPL under their breeding and maintenance protocol. The foster mothers will then be humanely killed. In the rare case the user does not have the adequate protocol, the animals will remain under our license on a breeding and maintenance protocol (protocol 4) for a maximum of one year.

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

Even though Protocol 2's severity (Embryo Transfer) is moderate and all animals undergo surgical embryo transfer, however we are expecting no more than 5% of the animals showing (adverse effects) complications due to the surgical procedure that would exceed severity limit. In very few cases, clinical signs can be observed in the initial post surgical period following the embryo transfer procedure. Some of the adverse effects that could occur are discomfort, pain, distress, impact on the mobility, its food and water intake, resulting in weight loss. In rare cases, embryo transfer surgery can result in the wound reopening causing pain and potential risks of infection, In this circumstance the animal will be anesthetised again and the wound cleaned and closed. If an animal is showing any of the previously mentioned adverse effects after embryo transfer surgery, it will be monitored by competent staff and a decision will be made whether it can be treated in order to make full recovery. If no appropriate measures can be applied to ameliorate the condition of the animal, it would be humanely killed. In all cases, an animal showing signs of discomfort or pain exceeding protocol severity, will be humanely sacrificed.

Expected severity categories and the 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 - severity Mild - 65%
Mice - severity Moderate - 34% - 1% sub threshold
Rats - severity Mild -75%
Rats - severity Moderate - 25%

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-vitro assays cannot adequately model the complete array of molecular, cellular, physiological and behavioural interactions necessary to fully understand how genetic modifications result in normal or abnormal processes. The use of animals is therefore compulsory in translational research for the relevance of the experiments and data, as Genetically Altered (GA) mice and rats are used as disease models in order to recreate particular biological pathways. Animal use is core to the activity of the transgenic facility and more so to achieve our objectives described in the plan of work.

Most users requesting our services are users that imperatively need to work with live models in order to carry out basic/translational research. Users will have directed previous studies to eventually require the need to generate a GA animal. The use of mice or rats become essential as well as the breeding of the colony in order to be able to carry out valuable relevant experiments.

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. However, users, due to the nature of their research, will have justified the need to be generating a GA animal in order to conclude initial results.
Such details will be expected in the justification for the animals' use to be reviewed by the AWERB.

Therefore, as the transgenic facility is planning on using this project licence to provide a service, animal use is compulsory. However, we will always make sure that the generation of a particular strain can be avoided by consulting and seeking our own database but also other facilities for similar existing strains to avoid duplications. We will constantly try to refine our work by improving methods and training, through regular assessments with help of local NVS and NACWOs.

Why were they not suitable?

Animal work is essential because many of the effects of biological processes under investigation involve complex physiological pathways that cannot be reproduced in vitro. The use of Embryonic Cells and synthetic DNA/RNA in-vitro can only provide results to a certain point. To confirm their impact on the genome they have to finally be used in-vivo to enable conclusive results. Mice/Rats will be used because many transgenic and knockout mutant strains have been created as disease models (Bedell et al, 1997).

Reduction

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

numbers consistent with scientific objectives, if any. These 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 provided are based on our own experience over the past 25 years and more specifically from our records collected for the past 5 years to more accurately reflect on the current workload. We have looked at the number of animals used in our previous PPL and have taken into account the reduction due to the COVID pandemic and alternatives to animal experimentation (such as computational research and in-vitro experiments). More importantly, we estimate the number of animals across all procedures, cryopreservation, rederivation, IVF, and the number of projects done in the passed 5 years, including a readjustment regarding 2020, when project demands have slowed down significantly.

The different parameters taken into account are the line, the background, the number of embryos obtained after superovulation, the % of fertilization, the % of unusable embryos, the viability of the embryos in culture ex vivo. All these parameters together allow us to build up reliable data to give the most accurate number of animals used in each protocol and assuring these numbers can be adequately fitted on a case by case basis.

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

All experiments are scaled , according to past experience (30 years) . The use of hyperstimulation and improved superovulation protocols has enabled us to reduce the number of females used as embryo donors . Improved cryopreservation techniques have enabled us to reduce the number of males and females used to securely cryopreserve genetically altered lines. The constant regular success of IVF procedures have also allowed us to adapt and reduce the number of embryo donors when records are showing a high quality (quantity, mobility) sperm previously cryopreserved.

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

Any animals the facility uses and/or humanely kills are offered to all users for tissue sharing. The facility holds a database of all mice and rats that have been imported or made on site , this database is checked before we undertake any experiments to generate or import Genetically Altered Animals (GAA). The facility is also a member of a transgenic community and Mouse locator which enables us to request strains of GAA from the worldwide community to avoid unnecessary experiment repetition.

Refinement

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

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

As a transgenic facility, our role is to provide a service and respond to a specific demand from users. We're using small rodents such as mice and rats because they are the most common species used in research in order to provide relevant basic/translational research. >95% of the work carried out using this license involves the use of mice models. In almost all cases, the generation of a new strain will require the use of Wild Type (WT) females such as for mice: C57Bl/6, CBA or B6CBAF1 and for rats: Sprague Dawley, Brown Norway, Wystar Kyoto (WKY) or Spontaneously Hypertensive Rats (SHR)... These animals will be purchased via a specialized breeding establishment. In very rare cases, the user will be providing embryo donor female, already genetically altered, to try and create multiple alterations within one already existing strain. Therefore, these females will generally be bred onsite and transferred to the facility to proceed with the embryo injections.

When alternatives to the use of animal models cannot be met, the facility guaranties to use the most up to date technique to generate genetically altered animals. The most reliable method is via transfer of embryos into the oviduct or uterus, which requires a surgical procedure (previously described in *Project Harms* section) . It is the procedure that offers the highest rate of embryo implantation, an aspect that is primary in the success of a project. It is primordial to consider that every possible embryos injected could result in being a positive founder, so a high rate of implantation is indispensable for the success of projects, avoiding re-injecting / rederiving again and therefore comply to the 3Rs. Non surgical procedure in order to implant embryos will rarely be used as firstly it is not viable for the stage of embryos we predominantly use and more importantly it shows a much lower percentage of implantation compared to surgical embryo transfer.

According to the procedure, the use of animals is not always necessary. When it comes to import and rederive already existing strains, this is mostly performed using frozen sperm/embryos, instead of importing live animals that would involve stress and subsequent rederiving, in order to comply to high standard health status. Generating a line from embryonic cell injections will not require embryo donor females as it's easier and more reliable to purchase an accurate number of good quality embryos at the right stage from specialized companies than ordering animals to obtain embryos that will need culturing, increasing the loss rate. It comes at a financial cost but it is more reliable. Only the generation of GA animals via IVF or single cell injection will require the facility to order females as embryo donors, that will need to be superovulated.

Some work will be carried out at the embryo stage of development, where embryos will be harvested, but this will still be using mice or rats having undergone the protocols listed above, and that will be humanely killed. In this case, pregnant female mice/rats are sacrificed before 2/3 of the gestation and the embryos harvested. However, these procedures are carried out for the needs of specific experiments and not considered as an alternative to using animals.

Breeding and maintenance of genetically altered animals are generic protocols where no adverse effects are expected. most animals kept under Breeding will show no adverse phenotype unless specified by the user, in which case the animal will be transferred under the user's project license.

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

In order to assess the functions of genes it is necessary to use live animal models as less

sentient species or cellular systems will not provide a full insight into the interactions of multiple physiological systems affecting whole body phenotypes. Rodents are the least sentient of mammal in which transgene technology works reproducibly, and are the most frequently used species as many different models of disease exist. Mice are the lowest vertebrate group that can be successfully used for basic/translational research. Even though zebrafish can be used and can show positive outcome when it comes to basic/translational research, investigation by the users will have been done to justify and validate the relevance for the use of mice/rats against other less sentient species through their own license. In addition, the mouse and rat genomes have been effectively mapped and show high similarities with the human genome. This information can improve our understanding of inheritance and inform future efforts to pinpoint the precise location of genes on individual chromosomes.

As a service license specialised in the generation of genetically altered murine lines, there is a protocol in place and paperwork required prior to acceptance of work. But this is paired with users having justified, after thorough justification through their own license, that the use of rodents was indispensable for the specific research being practiced.

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

Embryo transfer is the only "under 15 minutes recovery surgery" undertaken. It is performed by highly skilled and experienced technicians. Throughout the years, the technique has improved significantly. The use of gas anaesthesia in place of injectables, the use of an anti inflammatory drug combined with an analgesic, the constant improvement of the aseptic techniques as well as the recovery equipment (warming chamber) has led us to perform a surgery of a very high standard. Animals are making a full recovery within 30 minutes and are monitored by the personal licencee (PiL) in charge. Cages are labelled ensuring that the animal house staff monitor the animals beyond recovery. The animals are monitored the whole time throughout surgery, kept in warming chamber until they recover full mobility (usually taking from 5 to 30 minutes). Once recovered, the animals are put back in their cage, usually housed in pairs if possible with enviromental enrichment, and back in the rack in the animal room. PiLs will monitor the animals after surgery, paying attention to post traumatic injuries (risks of open wound) but also their mobililty, weight loss, signs of pilo-erection, possible hunched back. Animal technicians will then monitor the animals on a daily basis, once in the morning and once in the evenings and routinely when cages need cleaning, where animals are handled and thoroughly looked at. Cages will be labelled with a "to be monitored" label so that all staff can be aware and check routinely. To minimise pain and distress, the enrichment inside Individually Ventilated Cages (IVC) is optimised with tissue, chew sticks, cardboard houses and tunnels. Pregnancy will be checked after 7-10 days and the state of the wound will be assessed again, expecting it to be fully scarred by then. Animals will be group housed where possible, except in cases with justification (ie Stud males). Infections almost never occur (less than 5%). In the very rare case of a complication (for instance, a wound reopens), the animal will be assessed by the relevant competent member of staff and a decision can be made on whether care needs to be administrated to the animal, respecting the severity of the protocol (embryo recipient, protocol 2, severity "moderate") or if the animal needs to be sacrificed due to injury exceeding severity. The animal can then either be anesthetised again and given analgesics in order to perform the necessary procedure for the animal to make full recovery or the animal will be humanely killed.

Regular assessments ensure that competent members of staff can suggest any

improvements of the technique.

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

We consult websites that have information and regular updates regarding the 3Rs such as:
NC3Rs: www.nc3rs.org.uk

Consultation of new publications is another tool:

Pubmed: www.pubmed.ncbi.nlm.nih.gov

National Library of Medicine: www.ncbi.nlm.nih.gov

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

Regular seminars are organised within the institute in order to help users keeping informed about advances in the 3Rs, on general aspect as well as specific improvements within our institute. As PPL holders, it's our responsibility to keep ourselves up to date with the evolution of the 3Rs and apply ideas for better practice. Also, we are regularly assessed to update our CPD, which involve meetings with vets and NACWOS. These assessments help guiding us towards better practice and improvement of our procedures.

We consult websites that have information and regular updates regarding the 3Rs such as:

NC3Rs: www.nc3rs.org.uk
Consultation of new publications is another tool: Pubmed: www.pubmed.ncbi.nlm.nih.gov
National Library of Medicine: www.ncbi.nlm.nih.gov

34. Neuromuscular and neurodegenerative disorders: pathogenesis and therapy

Project duration

5 years 0 months

Project purpose

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

Key words

Therapy, Pathomechanisms, Neuromuscular diseases, Neurodegeneration, Animal Models

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

To i) investigate the disease mechanisms that result in debilitating and often fatal neurodegenerative diseases and diseases that affect the neuromuscular system, motor neuron diseases, inherited peripheral neuropathies, muscle disorders such as Inclusion Body Myositis (IBM), as well as forms of dementia (frontotemporal dementia) which manifests in ALS/MND and multisystem proteinopathy.

ii) to identify and develop novel therapeutic strategies to alleviate or cure these neurodegenerative diseases and diseases of the neuromuscular system, and for which there is currently no effective disease modifying therapy.

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

Why is it important to undertake this work?

This project will investigate disorders of the neuromuscular system, and includes diseases of the brain, peripheral nerves and muscle. The project will investigate a number of diseases that affect motor neurons, nerves and muscles including Motor Neuron Diseases such as Amyotrophic lateral sclerosis (ALS) and Kennedy's Disease (KD); peripheral neuropathies such as Charcot-Marie-Tooth (CMT), as well as muscle disorders such as Inclusion Body Myositis (IBM). MND is now recognised to be part of a disease spectrum that can affect motor neurons alone, or in combination with muscle, or other neuronal populations in the brain, causing specific forms of dementia, in particular Frontotemporal Dementia (FTD).

Despite significant improvements in our understanding of the underlying mechanisms of these debilitating and often fatal disorders, there is still no cure or effective treatment for these diseases. The development of effective therapies for these diseases therefore remains an imperative in the field.

To achieve this goal, we need to advance our understanding of the pathological mechanisms that play a role in these disorders, in order to identify, develop and test novel disease modifying therapeutic strategies for these disorders.

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

The outputs of the work undertaken in this project include:

new data and knowledge in the field of neuromuscular and neurodegenerative diseases, including IBM, ALS/MND, KD and FTD;

publications and presentations and, where appropriate, filing of new patents;

identification and development of candidate compounds with the potential to be progressed to clinical trials,

development of novel therapeutic approaches to therapy, particularly in ALS/MND.

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

The outputs of this project will benefit a number of groups including:

other researchers working in the field of neuromuscular and neurodegenerative diseases, in particular, in IBM, MND/ALS, FTD and KD;

the pharmaceutical industry;

our research group, in particular early career researchers and graduate and undergraduate students;
clinicians working in the field of neuromuscular and neurodegenerative diseases, in particular, in IBM, MND/ALS, FTD and KD;

patients affected by these diseases and their families/careers.

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

The outputs of this work will be maximised in a number of ways, including:

by collaboration with academic colleagues, within our University as well as national and international colleagues

by collaboration with industry;

by dissemination of our findings including publication in open-access journals, presentation at scientific meetings, and by presentation to patients at patient information days;

by making resources available to other researchers (e.g., data, animals, cells, tissues).

Species and numbers of animals expected to be used

- Mice: 12,500 total
- Rats: 4,000 total

Predicted harms

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

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

In this project we will mainly use mice, but in some cases, rats. Most of the mice we will study will be genetically modified to model aspects of the human diseases we are studying. As these diseases usually manifest in mid-late age, we will examine mice at various ages, including during development, to help identify the early underlying causes of disease, as well as in adulthood when the disease phenotype and signs begin to appear as these can be used to assess the effects of therapeutic interventions on disease onset and progression.

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, including potential therapeutic compounds, by injection using standard routes (intravenous, subcutaneous, intraperitoneal, intravenous). Where administration is required for prolonged periods, when the characteristics of the substance permits, animals will be treated orally by inclusion of the substance into their drinking water or food. In cases where this is not possible, the animals will be surgically implanted with slow-release devices such as a mini-pump. These animals will experience some discomfort after surgery and some mild to moderate pain which will be treated with analgesics.

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.

In order to test the efficacy of potential therapeutic compounds, the animals will be monitored longitudinally by assessment of body weight and assessment of their

neuromuscular phenotype, for example using a grip strength meter. These assessments result in no adverse effects or distress, and at the end of the test the animals will be returned to their home cage. In some animals, the neuromuscular phenotype will be assessed by terminal, physiological measurement of their maximum twitch and tetanic muscle force under anaesthesia; at the end of these experiments the animals will be humanely culled.

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

Most procedures in this project are mild or moderate, and based on our previous experience with the approaches to be used, we expect there to be few adverse effects. Some mice will have minor surgery, for example to implant a device under the skin that can release a medicine slowly or that can stimulate nerves. They are expected to recover quickly and will be given painkillers and post-operative care just like people recovering in hospital.

Some genetically modified mice will express mutations that are known to cause a neuromuscular or neurodegenerative disease in humans, which can result in deficits such as muscle weakness - a key characteristic of Motor Neuron Disease and other muscle wasting disorders. In such cases, these deficits are usually restricted to the hindlimbs, and may include dragging of a paw and/or gait abnormalities due to muscle weakness. In such cases, the animals will be provided with easy access to food and water, for example by providing a soggy diet within the home cage. In some cases, when the disease manifests it results in weight loss. In order to monitor for weight loss, these mice will be weighed weekly and if a decline in body weight is recorded, they will be weighed daily to ensure that weight loss does not exceed 15% of maximum, in which case they 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)?

The majority of procedures in this project will be mild or moderate:

- Mice: 75% mild, 25% moderate
- Rats 50% mild, 50% moderate.

For breeding and maintenance of GA Mice 10% will be mild, 10% moderate and 80% 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?

Explain if you will be able to replace any part or all of your proposed animal use during the course of this programme of work.

It not possible to achieve all of the objectives of this project without using animals as neuromuscular and neurodegenerative diseases such as ALS/MND involve highly complex interactions between different neurons and other non-neuronal cells within the brain and spinal cord, as well as cells such as muscle cells; it is not possible to model these highly complex interactions in culture or by computer modelling. Furthermore, many of these disorders manifest on a background of aging and/or changes in the immune system, which is difficult to model in culture.

In addition to in vitro models, several non-protected species, including fruit flies and nematodes have been used to generate transgenic models of neuromuscular and neurodegenerative diseases.

However, although these models may be of utility for some key questions (e.g., genetic screens) they have significant limitations for complex diseases such as ALS, particularly for the development of new therapeutic approaches. For example, in fly models of ALS, the expression of ALS-causing mutant human SOD1 (Superoxide Dismutase 1) does not induce motor neuron degeneration, making the identification of neuroprotective agents difficult.

However, where possible we will use cell culture models to address the key objectives of this project. We are currently optimising a novel culture model of the neuromuscular junction which involves the use of human stem cell-derived muscle cells and motor neurons and glia – almost completely replacing the requirement for animals in this model of the neuromuscular junction. Indeed, we are increasingly making use of human derived stem cell models of human disease, thereby reducing the need for animal experiments, which still however remain essential as modelling of highly complex systems and circuits such as the neuromuscular system is not possible in vitro. The use of human cellular models of disease is likely to become an increasing major focus of our work during the course of this project.

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

We will use non-animal alternative approaches in this project including in vitro methods such as cell culture. In our experiments we routinely use a variety of cell culture models including cell lines, mouse embryonic stem cells and primary cultures of murine cells (motor neurons, glia and muscle cells), as well as human iPSC-derived cells. These models have been invaluable in identifying new pathological pathways and for undertaking initial drug development studies. Over the coming years we also plan to continue to use these cell culture models to help identify novel biomarkers of disease and drug/target-engagement.

We have also considered non-protected species such as fruit flies and nematodes since several transgenic models of neuromuscular and neurodegenerative diseases have been generated. However, although these models have been shown to be highly valuable for some key questions, in particular for genetic screens, they have significant limitations for complex diseases such as ALS/MND, particularly for the development of new therapeutic approaches. For example, in fly models of ALS/MND, the expression of ALS/MND-causing

mutant human SOD1 does not induce motor neuron degeneration, making the identification of neuroprotective agents difficult.
In addition, as our overarching goal is to develop disease modifying therapies for neuromuscular and neurodegenerative diseases, we routinely compare our findings to available human data as well as our own studies on post-mortem tissue from affected patients.

Why were they not suitable?

Cell models are suitable as non-animal alternative models of neuromuscular and neurodegenerative diseases as indicated above.

However, although non-protected species such as fruit flies and nematodes have been used to generate transgenic models of neuromuscular and neurodegenerative diseases such as ALS/MND they have been shown to have significant limitations for complex diseases such as ALS/MND, and their utility in the development of new therapeutic approaches is limited. For example, in fly models of ALS/MND, although the expression of ALS/MND-causing mutant human SOD1 alters motor axon outgrowth, it does not induce the defining characteristic of this disease - motor neuron degeneration.
They are therefore of limited use in studies which aim to identify the molecular pathways that underlie motor neuron degeneration or in the development of novel therapeutic strategies.

Reduction

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

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

Mice bred in Protocols 1 and 2 will be subsequently used in Protocols 3, 4 and 5.
When assessing the numbers of animals we will use, we use an experimental design that includes consideration of the following:

Group size: these will be set based on published guidelines and existing data from previous work undertaken by this group using similar protocols, models and strategies for more than 30 years. For many of the experiments that will be undertaken in this project, consensus guidelines for how to conduct the investigation and minimum numbers of animals required per experimental group have been established and published, based on the review of multiple studies, from large numbers of investigators, over many years. For example, see 'Guidelines for preclinical animal research in ALS/MND: A consensus meeting' (Amyotrophic Lateral Sclerosis. 2010;11(1-2):38-45). We will adhere to these guidelines, to which our group contributed, as these are accepted by funders and researchers in the field and are based on the analysis on 20 years+ of publications of similar studies.

Experimental Group selection: typically, this will include i) male/female cohorts; ii) wild type controls and disease-model groups; iii) treated and control (vehicle)- treated cohorts; iii) typically, two dose levels of test compound.

Controls: For experiments investigating pathological pathways in animal models (Objective 1), the control groups will be i) non-transgenic wildtype littermates to control for assessment of genetically modified mice or ii) uninjured or non-pharmacologically treated littermates for assessment of injury models or pharmacological models, respectively.

In the case of Objective 2 (Development of therapeutic strategies), typically there will be three control groups: non-treated wildtype animals, treated wildtype animals and untreated disease model animals. Thus, when testing the effects of Drug "X" the experiment will consist of 4 cohorts: i) Model-treated; ii) Model-untreated; iii) WT-treated; iv) WT-untreated.

For physiological testing of neuromuscular function, 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 are larger (n=20 animals per group/sex/time point examined). For some experiments groups must be sex specific as disease progression often depends on gender (e.g., SOD1 mouse). Animals will be randomised to treatment groups and the experimenter blinded - usually with the aid of a colleague not involved in the study, both at the time of treatment allocation and at the point of assessment.

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

We have designed our experiments according to the NC3Rs ARRIVE Guidelines and the PREPARE Guidelines (Smith AJ et al. PREPARE: guidelines for planning animal research and testing. Laboratory Animals. 2018;52(2):135-141)

The experiments described in this project have been designed in consultation with statistical advisors. In all aspects of the project, measures will be undertaken to minimize the number of animals used wherever possible. For example, tissues from individual animals will be shared between group members and collaborators, so that an individual animal can be used to support experiments undertaken by several researchers, thereby reducing animal use 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 variability by careful phenotyping of the animals. For example, longitudinal analysis and phenotyping of SOD1 mice that model MND has shown that the disease progresses faster in male mice than female mice - resulting in the necessity to assess both male in female mice in preclinical testing of potential therapeutic compounds. Similarly, phenotyping has revealed that some behavioural assessments e.g., grip strength and rotarod testing have an inherent variability and therefore a cohort size must be set to account for this variability to obtain reliable data (min n=12/group). Some assessments are also known to be experimenter-dependent, and so these tests will be carried out by the same experimenter throughout the study to minimise variability.

In this project, we will minimise the numbers of animals used by maximising the data obtained from each animal, for example, by undertaking longitudinal tests or physiological analysis in animals in which the relevant tissues will be removed at the end of the experiment for post-hoc molecular and histopathological analysis. Wherever possible, we will make use of stored tissue available in existing biobanks and will contribute tissue from animals used in our experiments to these biobanks for future use by ourselves and

colleagues working on related subjects.

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 with BSU staff to ensure efficient breeding of our colonies. We have a lab manager with experience in managing animal colonies and overseeing breeding programmes, who will monitor animal use and the breeding colonies used in this project.

Where possible, we will reduce the number of animals used in this project by using existing ex vivo material stored in tissue banks or by using surplus stock.

To optimise the number of animals we use in this project we typically undertake pilot studies. Usually, pilot studies will first be undertaken in vitro, using cell models to identify pathological pathways/targets and in experiments testing therapeutic approaches, to establish target engagement, to determine a dose response curve and establish indicative concentrations to be used in vivo. In vivo pilot studies will consist of experimental groups of smaller numbers than in the definitive efficacy study, e.g., in physiological experiments examining muscle force, n=3 treated and 3 untreated mice that model the disease will give an indication of efficacy. However, due to the known variability and sex effects of many transgenic models, the larger numbers are required in the definitive study to undertake statistically robust analysis to demonstrate efficacy.
In this project, we will minimise the numbers of animals used by maximising the data obtained from each animal, for example, by undertaking longitudinal tests or physiological analysis in animals in which the relevant tissues will be removed at the end of the experiment for post-hoc molecular and histopathological analysis.

At the end of the experiments, we will harvest as many tissues as possible at post-mortem and if these tissues are not required for immediate examination, we will freeze the tissues 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.

A large proportion of 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 therefore dissect 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 numbers of animals to be used in this study. Mice expressing mutations that are known to be involved or cause the diseases under investigation in this project have already been created and new mice are in development.

Wherever possible we use in vitro models, e.g., -cultures of primary motor neurons and glia from genetically modified rodents modelling disease e.g., SOD1 mice, to examine disease mechanisms and test potential therapeutics, prior to validation in vivo. Increasingly we are also using human iPSC models of disease which completely avoids the need for animal use. We also use models with as mild a phenotype as possible to test specific questions e.g., milder SOD1 strains with fewer transgene copies, which have a milder form of the disease than high copy number strains.

It is also possible to model aspects of pathology which avoid 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. In this project we will use the model with the mildest phenotype that model aspects of disease pathology. We will minimise harms during surgery by undertaking the mildest injury required to meet the scientific objectives and induce the required phenotype e.g., lesions will be unilateral and 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 base of the home cage following surgery or when the disease phenotype reduces mobility (e.g., with SOD1 mice).

A major focus of this project is the development of novel therapies for neuromuscular and neurodegenerative disorders. Therefore, using the most appropriate disease model, we will undertake pre-clinical testing of potential therapeutic agents, including pharmacological, genetic therapies and cell replacement strategies. These will be given to the animals via the least invasive route to minimise distress to the animal and will be determined by factors such as the chemical characteristics of the test compounds, site of pathology being targeted, or bioavailability. Animals will be treated by mouth, injection, or through food, or by the use of implanted mini-osmotic pumps for long term treatment.

As the human disorders we are studying typically manifest on an adult or aging background, it is necessary to study the animal models throughout the disease course, including up to humane endpoints, particularly to establish the effect of therapeutic approaches on disease onset, when disease signs first appear, as well as lifespan, as the human diseases we are studying, such as MND, result in premature death, and human clinical trials rely on survival as an outcome measure of efficacy. We will monitor animals in such studies carefully to ensure they are maintained in the best physiological state, with regular assessment of the appearance, body weight and disease signs such as muscle weakness. As disease progresses, animals will be assessed daily. For diseases such as MND, 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 the majority of MND 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 too 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 aging background.

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

We aim to refine the procedures we use in this project in order to minimise the welfare costs for the animals. For example, in models of disease, we closely monitor the animals for signs of disease onset, after which the monitoring becomes more intense, such as assessing the animal twice daily for adverse effects such as changes in weight, dermatitis, piloerection, paleness.

In some cases, it may be possible to reduce the duration of the experiment by studying onset of symptoms as an outcome, or disease severity at an earlier stage of the disease.

For animals that have undergone surgery, a post-operative care plan is put in place, which includes pain management and assessment of recovery.

As some of the experiments will include assessment of animals as they age, to account for the aging background that these disease manifests on, we will ensure that the group sizes in these experiments is sufficient 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 if the adverse effects do not resolve, the animals will be humanely killed.

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

We have considered the PREPARE guidelines when planning the experiments described in this project, which build on the ARRIVE guidelines which we have previously followed (Smith AJ et al; PREPARE: guidelines for planning animal research and testing. Laboratory Animals. 2018;52(2):135- 141).

We have also based the design of out therapeutic testing experiments on published guidelines for studies undertaken in mice that model MND (Ludolph et al, 2010: Guidelines for preclinical animal research in ALS/MND: A consensus meeting. Amyotroph Lateral Scler. 11(1-2):38-45) which are accepted in the field by researchers, funders and journals as the standard experimental design for preclinical research in MND.

We will access guidance and publications from 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 NC3Rs website, and we are signed up to the NC3Rs newsletter. In addition, we are signed up to receive regular newsletters from the organisation 'Understanding Animal Research'.

Our oganisation is committed to the principles of the 3Rs to minimise harm and discomfort to the animals and encourages and supports implementation of advances in the 3Rs by all Licence Holders. Our Biological Services website has a dedicate 3Rs page which notifies users of news in the 3Rs.

35. New treatments for renal and liver disease

Project duration

5 years 0 months

Project purpose

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

Kidney, Diabetic, Treatment, Renal, Liver

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?

The aim of this project licence is to develop novel treatments for patients with renal or liver 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?

Renal diseases remain devastating illnesses with unacceptably high rates of mortality and morbidity worldwide. The kidney is a complex organ made up of over 26 different cell types

and plays a critical role in toxin elimination, pH balance and hormone production. Acute kidney injury (AKI) is a sudden loss of kidney function whereas chronic kidney disease (CKD) is a progressive loss in kidney function over a period of months or years. When kidney function falls below a certain point, it is called kidney failure. Between 8 and 10% of the adult population have some form of kidney damage, and every year millions die prematurely of complications related to kidney diseases. Kidney dysfunction is a disease area that impacts many lives but where there are currently limited treatment options available. Once a patient reaches kidney failure, a kidney transplant is necessary to prevent death. As insufficient kidneys are available to meet demand only a small proportion of patients undergo a kidney transplant operation. The remainder must undergo dialysis, where a machine is used to perform the function of the kidney to clean the blood. Even with timely dialysis, the death rates in patients with kidney failure vary from 20% to 50% over 24 months.

Non-alcoholic fatty liver disease (NAFLD) is the most prevalent chronic liver disease worldwide and is often found in patients with type 2 diabetes. There is no approved treatment for NAFLD. Fatty liver disease and inflammation in the liver will eventually lead to cirrhosis (scarring of the liver). A patient with liver disease is also at a higher risk than the general population for developing a form of liver cancer called hepatocellular carcinoma (HCC).

Clearly novel approaches for the treatment of liver and kidney diseases are required to help the many patients suffering. In work under this licence we aim to identify new treatments for CKD, AKI and NAFLD. As we pursue our goal of finding new therapeutic approaches will also uncover new insights into disease progression and learn more about the process of repair from injury in the liver and kidney. Wherever possible scientific discoveries and developments arising from this licence that are not commercially sensitive will be published to the benefit of the wider scientific community.

Based on our past record we would expect to deliver 3-5 potential new drugs into clinical development during the period of this licence. Whilst not all of these will be successful, as we move to more novel and sophisticated approaches to clinical trials, it seems reasonable to predict that approximately 20% of the nominated compounds will provide significant benefit to patients with renal or liver disease.

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

The benefit of this project will ultimately be the introduction of new and improved treatments for the management of kidney and liver disease. This will be achieved by progressing novel candidate compounds to clinical trials and discontinuing ineffective approaches. Other benefits include publications, presentations and patents filed. Our research will also advance our basic biological understanding of the kidney and liver in health and disease.

For liver disease specifically, work carried out on this project licence will only support experiments that will be carried out in the laboratory using cells and/or tissues harvested from mice or rats. Drugs that look promising in those experiments will be taken further in work that will take place outside of this project licence.

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

In the short term other researchers within the field and the pharmaceutical industry will

benefit from increased knowledge and learning in which therapeutic approaches might be the most effective and which are not. In medium term (10 years) patients and clinicians will hopefully benefit from the launch of novel therapies for renal and liver disease, that will provide therapies for diseases where there is currently no specific treatment such as NASH or AKI, or provide more effective therapies, or therapies with reduced side effects for patients with CKD. In the long term (20 years) society will hopefully benefit from the decreased health burden that will result from the development of new drugs and technologies.

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

Wherever possible scientific discoveries and developments arising from this licence that are not commercially sensitive will be published to the benefit of the wider scientific community through publication in open-access journals and presentation at scientific meetings. When not commercially sensitive resources, such as data, reagents, tissues will be made available to other researchers. For example detailed characterisation of animal models used for pre-clinical drug testing has previously been be shared within the renal community to accelerate drug development. We will aim to publish approaches to therapy that are ineffective or have developability challenges to prevent this work being repeated by other companies or researchers.

We will share good practice with the local Animal Welfare and Ethical Review Body (AWERB) and also within internal and external forums that bring together researchers utilising animals for scientific purposes from across different disciplines.

Species and numbers of animals expected to be used

- Mice: 7200
- Rats: 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.

We will use rats and mice during this program of work. Some animals will be genetically altered to modulate a specific gene, or to produce a specific disease state. We have chosen rats and mice because 90% of the genes in rodents are shared with humans and there is extensive literature characterising mouse and rat physiology. Many models of human disease have been developed in mouse and rat and share many of the characteristics seen in human disease. Because of these similarities we can be more confident findings that we make in mice and rats will also apply to humans.

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

Upon arrival to our facility the mice or rats are transferred into clean Individually Ventilated Cages (IVC). The cages are maintained at the appropriate temperature and humidity for the species. Cages are cleaned at least once a week and water and food is checked daily. Animals are left to get used to to their new home surroundings for at least 6 days before any experimental procedures are performed.

Mice and rats are typically housed in small groups as they are social animals. On occasions animals may be singly housed e.g. on occasions male mice naturally fight with each other so may be housed singly for welfare reasons.

Mice and rats will be used either to harvest cells or tissues or to evaluate novel drugs that are potential new treatments for kidney disease.

In efficacy studies a disease state will be induced so that the effectiveness of novel drugs in improving kidney disease can be assessed. Disease will be induced by either :- genetic alterations, modified diets, administration of drugs with the potential to damage the kidney or surgical procedures under general anaesthesia (i. removal of one kidney, ii. obstruction of urine flow, iii. temporary blocking of blood flow to the kidney). The surgical procedures will be carried out as follows i. For the removal of one kidney, a 1-cm incision will be made in the flank of the animal, the kidney will be exposed and surgically removed, and the incision will be closed with sutures. The surgical procedure is expected to take approximately 20 minutes. ii. For the obstruction of urine flow, an incision will be made in the flank of the animal and the ureter will be tied off using a fine piece of silk, the ureter is the canal that carries urine from the kidney to the bladder. The incision will be closed with sutures. The surgical procedure is expected to take approximately 20 minutes. iii. For the temporary blocking of renal blood flow an incision will be made in the flank of the animal and a small clip put on the vessel supplying blood to the kidney. Blood flow will be stopped for typically 25 minutes (and never longer than 60 minutes) while the mouse is maintained under anaesthetic and body temperature is maintained by a heating pad. After that the clamp will be removed and the incision will be closed with sutures. For all the above, no experiment will be performed until full recovery of the animals.

Genetically modified animals are used that display phenotype that mimic many of the characteristics of patients with diabetic kidney disease. Namely these animals may experience weight gain, high blood sugar, excessive urination in combination with high blood pressure, depending on the genetic modification.

Irrespective of the method of inducing kidney damage the characteristics of the kidney injury will be determined in a small number of animals if not already known before proceeding to larger studies looking at the effect of potential kidney drugs. In these small pilot studies the animals are closely observed every day and body weights and condition of the animals are recorded to ensure that the animals are healthy. If kidney damage has been induced in a consistent manner this model of disease can then be used to test the effectiveness of novel drugs. Drugs can be dosed through the mouth, into the peritoneum (body cavity), in the fatty tissue, just under the skin or alternatively into the vein. Direct administration into the blood stream may require the surgical placement of a permanent cannula into a blood vessel. Where administration is required for prolonged periods, animals may be surgically implanted with slow release devices such as a mini-pump. The surgical subcutaneous implantation of a mini pump is carried out under general anaesthesia and involves making a small incision on the back of the mouse, typically between the shoulder blades, a small pocket is created under the skin and the mini pump inserted with the catheter tubing extending out from the incision site. The incision is closed using sutures or tissue adhesive. In some circumstances substances will be administered directly into the kidney - this procedure will be carried out under general anaesthesia with a surgical procedure to isolate the kidney by making a 1-cm incision in the flank of the animal and exposing the kidney. After the micro-injection into the kidney with a fine needle is completed, the surgical incision is closed.

Another method to administer a treatment into the kidney is to use ultrasound to visualise the kidney in an anaesthetised animal and then use a needle to pass through the skin and inject directly into the kidney. In all surgical procedures the animals are given post-operative pain killers and monitored closely for any signs of pain, infection, or other complications.

The effect of the potential treatment for renal disease on renal function is compared to function in an animal which does not receive the novel treatment. Renal function can be assessed by tests performed in urine or blood collected from the animals. Urine collection will be done in specially designed cages in which animals may be kept for up to 72 hours, alternatively a spot sample maybe collected through non-invasive means by placing the animals on a type of sand that does not absorb the urine and allows it to be easily collected, following spontaneous urination by the animal. Blood samples may be collected from a vein. Other measures used to assess the effectiveness of the drug treatment and in some circumstances understand how it is working include blood pressure and imaging under general anaesthesia.

At the end of the study the animal is killed, and the kidney and other tissues may be taken which can then be used for further investigation. This procedure is done under non-recovery anaesthesia where the animals will only be aware of the anaesthetic being administered and may experience mild distress and no pain.

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

Animals will experience mild, transient pain and no lasting harm from administration of substances by injection using standard routes and from blood withdrawal. The total number of injections an animal can receive is limited. The amount of blood that can be collected is limited in relation to the animals blood volume.

Mice will have surgery either to induce kidney damage or to administer drugs directly to the kidney or they may have minor surgery to implant a device under the skin that can release a medicine slowly. They are expected to recover quickly and will be given painkillers and post-operative care.

Some diseased animals will experience weight gain, high blood sugar, high blood pressure, increased drinking and excessive urination which will last for the duration of the experiment but will not reach a level that is likely to cause pain or discomfort to the animals.

Some animals may experience stress associated with isolation from single housing, such as when animals are housed in cages for urine collection.

Animals may experience transient stress associated with restraint for blood pressure measurement.

Animals which undergo changes in diet are not expected to experience distress but may result in obesity. Some diets may result in weight loss due to unpalatability. Animals will be placed onto normal diet should they lose 15% of their body weight.

Drugs maybe used to induce a disease state. Some drugs may damage cells within the

kidney. Other drugs may be used to damage insulin prodcuing cells within the pancreas. The loss of these cells will induce diabetes and in this way we can study kidney disease than is caused by diabetes. The use of certain drugs, which are used to induce a disease state, may cause transient weight loss after administration. Animals will typically recover the weight lost due to drug administration within 1 to 2 weeks.

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

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

Mice Non-recovery 2%
Mild 23%
Moderate 75% Rats Non recovery 2%
Mild 23%
Moderate 75%

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

- Killed

Replacement

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

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

Whilst we use in vitro (experiments performed in cells in the laboratory) or in silico (experimentation performed by computer methods) where possible to short list candidate drugs, for many projects assessments of efficacy in disease for lead molecules will need to be assessed in vivo (in animals). There is currently no in vitro or in silico system capable of simulating complex whole animal physiology and the complexity of the kidney made up of at least 26 different cell types. Metabolic and kidney diseases have a complex origin with multiple components interacting to manifest the disease. Many therapeutic agents target specific biochemical responses or physiological mechanisms that in vitro systems cannot fully replicate.

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

As an organisation we invest heavily in human tissue and cell based technologies for our renal research. For example we use human kidney organoids to test drug activity in certain projects. An organoid is a miniaturized and simplified version of an organ produced in vitro in three dimensions that mimics the key functional, structural and biological complexity of that organ.i We also use mouse precision cut kidney slices and isolated rat glomeruli (a sub-structure within the kidney that acts as to clean blood passing through it). These systems are used extensively for mechanistic and pharmacological studies prior to in vivo studies. We have also evaluated other organ-on-a chip technologies that are commercially available from companies such as Mimetas and hope to add these systems into existing workstreams following validation of these platforms.

Why were they not suitable?

Individual mechanisms can be probed in cells or complex 3D models, and we conduct extensive studies to characterise these as far as possible before conducting experiments in animals. However processes such as filtration through the glomerulus are hard to replicate plus we need the interaction between different organs such as kidney and heart or the interaction between the kidney and the different cells of the immune system.

Reduction

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

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

We typically run on average 3 studies per month, each study may have 40-60 animals per study.

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

All studies are designed to ensure that the minimal numbers of animals are used to achieve the question being asked. This is done with help and guidance from a statistician who is a maths expert who uses huge amounts of data to figure out how likely it is that something will happen or not. We also use the NC3Rs EDA (https://www.nc3rs.org.uk/experimental-design-assistant-eda) a free online tool that helps to check the minimum number of animals is used consistent with the scientific objectives of the experiment. We also consult the NC3Rs website for general advice on reduction.

Pilot studies will be run for new models to understand effect size and variability and these data will be used to calculate how many animals are needed to subsequent experiments. Good experimental design principles such as randomisation are incorporated into all experiments. All study designs are approved by a statistician.

All experiments are performed in accordance with Good Laboratory Standards (GLS). This standard sets the minimum laboratory requirements for all our research and development. This ensures that procedures and results are accurate, reliable, traceable and reproducible and where appropriate,comply with the appropriate regulatory authorities' legislation.

All experiments are performed in accordance with the PREPARE guidelines - Planning Research and Experimental Procedures on Animals: Recommendations for Excellence.

All research that will be published will be published in accordance with the ARRIVE guidelines - Animal Research: Reporting of In Vivo Experiments.

Where possible mixed sex groups will be used in 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 hope to optimise new imaging efficacy endpoints that will allow assessment of animals over time. This will mean several timepoints can be evaluated in the same animal and therefore less animals in total may be needed for a particular project. Where appropriate, samples from in vivo studies can be shared with collaborators to the maximise the scientific knowledge that can be gained from one study.

Refinement

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

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

Models are chosen based on the on the minimal pain and suffering to the animal in combination with the degree to which they faithfully replicate the disease process, or aspects of the disease process in humans. 90% of genes present in mouse and rat are also present in humans. Mouse and rat models of metabolic and kidney disease have been established by other groups and reported in the literature.
The majority of drugs used in patients today to treat diabetes and kidney disease have the same effects in rodent models of disease. The inclusion of mice enables us to use mutant or genetically modified animals for early hypothesis testing, target validation and humanization of target as necessary.

Novel drugs are tested in a very small number of animals initially (typically 2 to 3 per group) and only drugs that do not have unwanted side effects can be used in larger numbers of animals.

Some methods to induce kidney injury require a surgical procedure. Any animals that undergo a surgical procedure will be provided with pain medication prior to the surgery (and after surgery where required) and maintained in a warm environment until full recovery to minimise weight loss.

All animals will be kept in cages that have various forms of enrichment included, for example a cardboard house, sizzle nest, tunnels, chew stick. The temperature and humidity is kept within a specified range that is optimal for the animals.

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

Mice and rats have a well-defined biology with a highly characterised immune system. In non- mammalian species such as the fruit fly, is not possible to replicate the complex processes that underpin metabolic disease and kidney and liver dysfunction.

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

Environmental enrichment is provided in every cage. Enrichment provided such as paper houses, tunnel and chew sticks allow the rats and mice to have improved welfare and demonstrate natural behaviours such as sheltering, nesting, climbing and gnawing.

Pilot studies will be conducted for new protocols to ensure the methods used provide for the maximum animal welfare in relation to the experimental objective. We will also aim to implement new ways, as technology evolves, to further improve the welfare of the animal over the course of these experiments (e.g. by embracing non-invasive measurements).

We now implement non-aversive mouse handling methods on all studies. This involves holding the mice using a tunnel or cupped hands rather than picking up by the tail to reduce any anxiety induced by handling.

All animals will be acclimatised for 7 days from arrival before they undergo any experimental procedure.

We closely follow and implement the latest welfare guidelines and therefore handle animals in a way that causes the least amount of harm or stress to them as possible while conducting these experiments.

All surgery is performed in concordance with 2017 LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery. Animals will be given pain killers prior to surgery and afterwards.

For some compounds it is possible to administer them in the diet or drinking water and this has the potential to reduce animal handling in chronic dosing studies.

Use of acclimatisation to reduce stress response to restraint for example with blood pressure measurement.

To reduce male aggression some clean and dry (i.e. used but not soiled) nesting material is transferred from the old cage to the new cage during cage changes. It is known cage cleaning can disrupt social signals communicated through scent and thus disrupt the social hierarchy. Transfer of bedding material can prevent this and therefore decrease male aggression. Environmental enrichment such as partitions and tunnels can also reduce the prevalence of aggressive behaviour in group housed mice. The NC3Rs resource on reducing aggression between group housed males will be used to guide additional practices for reducing aggression.

In studies where the administration of tamoxifen to animals is required for the scientific purpose, the experimental design will be guided by "best-practice" resources produced through culture of care workshops organised within the scientific community.

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

We will use the PREPARE guidelines to assist with planning animal research. We will also use of web- based sources through the National Centre for the Replacement, Refinement and Reduction in Animals in Research (www.nc3rs.org.uk/experimental-design), ARRIVE (Animal Research: Reporting of In Vivo Experiment, guidelines for preparing publications; https://www.nc3rs.org.uk/arrive-guidelines) and Laboratory Animal Science Association (LASA) guiding principles documents for aseptic technique for any surgical procedures

(https://www.lasa.co.uk/current_publications/).

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

Subscribing to publications such as ATLA (Alternatives to Laboratory Animals) Journal: https://journals.sagepub.com/home/atla; NORECOPA newsletter

Having regular discussions with the Named Persons and animal technicians within the facility to review current approaches and whether there are any new 3Rs opportunities and subscribing to the internal 3Rs enquiry list.

Attending NC3Rs events and workshops

3Rs resources are available on the University's in vivo SharePoint site. Frequent visits to the National Centre for the Replacement, Refinement and Reduction of animals in research's website. The following websites are also consulted for practical guidance Laboratory Animal Science Association (LASA), Jackson lab IMSR (international mouse strain resource) repository http://www.findmice.org/participate, LASA 2017 Guiding Principles for Preparing for and Undertaking Aseptic Surgery and the Royal Society for the Prevention of Cruelty to Animals (RSPCA).

We also actively discuss and implement new 3R's initiatives and run a yearly 3R's competition, sharing information globally across different establishments. We actively set annual refinement goals each year within the department.

36. Mouse developmental genetics to study neurodevelopment and disease

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Neurodevelopment, Stem Cell Biology, Regenerative Medicine, Cerebellum, Brain injury

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

This project aims to identify and study the cellular and molecular mechanisms that regulate neural stem cell behaviours during development, adulthood and upon injury to the brain. Neural stem cells are specialized cells of the brain that are able to give rise to neurons and glia, cells of the brain that are crucial for its function. Neurons transmit information from the body to the brain and back to the body so we can run, smell or think, whereas glia are the supporting cells of the brain which help the neurons to function efficiently. Neural stem cell function is crucial during development to ensure our brain is formed properly and also later in adulthood to ensure our brain continues to function efficiently.

However, generally in the brain, our cells do not get replaced efficiently and cell loss after injury or disease is detrimental. This project utilizes the mouse brain, and specifically the cerebellum, a critical brain region that is important for motor, cognitive and social behaviour, basically how we move, think and interact with others, as a point of entry to study neural stem cells in mammalian systems. This understanding is the crucial first step to designing stem cell-targeted therapies that facilitate the repair and regeneration of brain cells.

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

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

Why is it important to undertake this work?

Brain injuries have devastating outcomes due to the loss of cells followed by inefficient repair. Currently, there are 1.4 million people that are living with acquired brain injuries and 40,000 deaths only from stroke annually in the UK, and an estimated 69 million people suffer from traumatic brain injuries and 14 million new incidents of stroke globally, each year. This highlights the desperate need to develop novel regenerative therapies. However, our knowledge of the stem cells in the brain that can facilitate repair, as well as the molecular and cellular mechanisms that regulate neural stem cell populations' behaviours, such as how they divide or make neurons are limited. Lack of this understanding impairs the development of stem cell-targeted therapies that are desperately needed to replace and repair the injured cells in the brain upon injury. Successful accomplishment of this project will provide the crucial knowledge to design therapies that can activate stem cells of the brain to make new neurons after injury. Additionally, the cerebellum a region of the brain that is critical for motor and cognitive functions develops later than the rest of the brain and therefore is susceptible to injury around birth. Indeed, cerebellar defects are the second leading risk factor for autism spectrum disorders.

Therefore, in addition to representing a powerful model to study neural stem cell biology, understanding the regenerative potential of the cerebellum has an impact on newborn health and development.

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

In the short term, the successful completion of this project will provide much-needed new information on the molecular and cellular events that govern stem cells during development, adulthood and upon injury to the mouse brain. In light of this information, we will be able to design and develop approaches to understand how these mechanisms are conserved in humans and how we can facilitate regeneration in the human brain. We will disseminate the results of this project throughout the project period via presentations at international conferences and publications in peer-reviewed journals. We anticipate attending at least one conference each year and publishing 3-5 peer-reviewed articles at the end of the project period.

Importantly, in the long term, this project will provide a springboard to utilize endogenous neural stem cells as therapeutic entry points for regenerative medicine in the brain.

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

In the short term scientific community, particularly groups of scientists who are interested in the fields of neurodevelopment, stem cell biology and regenerative medicine will benefit from the data and the tools generated as a result of this project. In the long term, this data will provide fundamental knowledge on the development of the brain, and particularly the cerebellum, identify and describe neural stem cells and provide tools/methodology to manipulate these cells. Furthermore, experimental models that utilise human cells to study the cerebellum are limited. The knowledge that will be generated as a result of the project will also inspire studies that will assess how conserved these mechanisms are in humans and enable the establishment of more efficient systems to study human biology. Finally, in

the long term, this understanding may also lead to the development of therapies to facilitate regeneration, in other words, new neuron production after injury to the brain, benefiting many who suffer from brain injury.

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

We will openly share all data, a detailed explanation of the animal work and procedures in all our publications publicly via open-access journals. We will present our unpublished findings at conferences with the greater scientific community. We will also openly share the resources generated prior to publications with collaborators upon mutual agreements in order to minimize duplication of efforts and facilitate the efficient distribution of resources within the scientific community. We will also disclose unsuccessful approaches and results on our websites or via publications when possible. The large/high throughput data sets such as genomics data generated as a result of this project will also be uploaded into public domains.

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.

The major goals of our studies are to elucidate how the mammalian brain and its underlying cellular and functional complexity are established during development, and how signalling factors regulate stem/progenitor cell behaviours in developing and adult organs during regeneration. Developmental processes and stem cell responses cannot be fully replicated with any of the existing tissue culture technologies since a complex set of cellular interactions must occur in a precise three-dimensional organization and over an extended time period. Furthermore, these processes and cellular behaviours cannot be modelled on the computer. Transgenic technologies provide ingenious ways of studying the control of gene expression and of following the consequences of altering or blocking specific gene products leading to the discovery of gene function. By analysing the outcome of such manipulations using various transgenic mice, we can address questions of how development occurs or diseases form. By being able to label and visualize normal and mutant cells amongst their neighbours, and following their development or response to injury, we can determine the cellular movements and interactions that underlie normal development and disease. This line of research is required to understand the molecular mechanisms that govern neural stem cells and establish the necessary knowledge required to develop future therapies against human disorders.

Our previous studies have highlighted that the neonatal mouse cerebellum (postnatal (days after birth) day 0 – 5) is highly regenerative and can recover from the loss of its neurons at birth, whereas the adolescent or adult mouse cerebellum loses this ability dramatically. In order to understand the age- dependent changes in the repair capacity of the brain, our research involves studying various stages, particularly early neonates (Postnatal 0 - 5 days) and early adulthood (4-8 weeks).

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

Experiments will involve the following procedures:

Breeding and maintaining of genetically engineered mouse models (~7-8 lines, that consist of cell type specific Cre/CreER and Flpo/FlpoER animals and R26 Cre/Flp dependent reporters, floxed alleles for key genes of interest. These genetic mouse lines allow us to label and follow cells of interest and their daughter cells). We will collect tissue for genotyping in-house. Optimize our breeding to maximize the use of litters and minimize the cages needed for maintaining our animals outbred.

Genetic inducible fate mapping: The majority of our work involves studying which cells do neural stem cells generate under different conditions. Experiments will involve Tamoxifen (a drug) injection to induce specific labelling of neural stem cells at multiple different ages based on the experimental paradigm. The majority of our time points are postnatal and we have not had adverse side effects of Tamoxifen at these doses/time points. Some of our experimental paradigms also involve transectional approaches that require the administration of Doxycycline (a drug) in the drinking water for defined periods that will allow us to achieve specific labelling of cells that would otherwise not be possible.

Injury models: Our work involves studying the stem cell responses to injury at various postnatal ages. We will use established injury models such as localized mild stroke models that are carefully designed to ensure animal welfare and minimise pain.

Irradiation to the neonatal mouse: A single dose (4Gy) cerebellum targeted (via specific collimators or body shielding) irradiation at postnatal day 1 to anaesthetized pups. The neonatal cerebellum can recover from this injury very efficiently and no visible behavioural effect has been observed. We also do not observe any immediate morbidity in the pups due to the procedure and no associated adverse effects.

Targeted stroke model (photothrombotic injury): Anesthetized mice are injected with Rose Bengal (a drug) and a cold LED light source is shined over the skull (upon incision) for 10-15 minutes. This causes local blood clothes and a mini stroke-like injury. This is a widely used and published model. We have optimized the parameters to ensure the strokes are mild, animals recover well and do not show obvious physical and behavioural damage upon injury. In the case of adverse effects, animals will be humanely killed.

Targeted cell killing: Using transgenic mouse lines, we selectively express diphtheria toxin receptor in the cell types of interest to allow their killing. Diphtheria toxin will then be injected (30 ng/g) and the response to injury will be assessed. The paradigms we will use involves the selective killing of small subpopulations within the brain and we do not observe mortality/ lack of fitness in these animals upon injury. Importantly, the dose of diphtheria toxin used for these experiments is low and is not harmful to humans, therefore experiments can be performed within specific biosafety requirements.

Intracranial (in to the brain) injection of cells or other biologicals (such as lentivirus): Cranial windows will be opened (Skin incision and drilling of the skull (adult animals)) and small volumes (1- 5ul) of cells or inactive lentivirus will be injected into target regions using a stereotactic frame that immobilizes the head of the animal. Proper analgesic regimens will be given. This is a well-established procedure and used in the neuroscience field for decades.

Intraperitoneal/subcutaneous injection of various chemicals: small molecules, BrdU, EdU. Tissue collection for data analysis: Brain tissue from various stages will be collected from all the above paradigms (except for 1) for downstream analyses such as histology or other molecular biology approaches. Estimated tissue collection times after the experimental procedures explained in (2-5) vary between 1-2 hours to up to 2 months after the procedure)

Fresh tissue collection for primary culture establishment/cell isolation from various ages.

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

Our research involves multiple cell ablation and injury models. Our previous research shows that neonatal mice recover from injury well and these procedures do not cause obvious defects in the cerebellar cytoarchitecture or mouse behaviour. On the other hand, the injury paradigms used in adult mice and other surgical procedures used at all ages may cause mild to moderate pain in animals until the experimental endpoint or briefly after the procedure, respectively. Adult mice that have undergone a surgical procedure will be killed within 4-8 weeks unless they do not show any severe complications, weight loss and signs of distress. Neonatal animals will also be closely monitored for their feeding behaviour and growth, as well as signs of distress. All animals will be immediately killed if they exhibit signs of distress.

Some of the mutant animals (generated as a result of genetic loss of function studies) may cause developmental abnormalities. We will utilize genetically engineered mice that bears Cre-lox (or Flpo- FRT) systems that are temporally controlled, when possible, to restrict the effect of the mutation on the cells of interest and time in order to minimise the systemic effects. When needed we will perform pilot experiments to assess the severity of the phenotype to determine humane experimental endpoints.

Importantly, we are looking forward to sharing the mouse lines, experimental models and procedures that we will establish during this project and the specimens and data generated from these experiments open with the scientific community. We believe that this will prevent duplication of efforts and increase the rigour and reproducibility of our and others' research while allowing refinement and reduction in our experiments.

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

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

Species	Severity	Percentage
Mouse	Mild	80%
Mouse	Moderate	20%
Mouse	Severe	0%

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?

A major goal of our studies is to elucidate how signalling factors regulate stem cell behaviours in developing and adult organs, and how do these behaviours change upon injury. Developmental processes, stem cell responses and injury response/regenerative processes cannot be fully replicated with any of the existing tissue culture technologies since a complex set of cellular interactions must occur in a precise three-dimensional organization over an extended period of time. Furthermore, these processes and cellular behaviours cannot be modelled on the computer. By analysing phenotypes in transgenic mice, we can address questions such as what a gene's function during tissue development and homeostasis or in disease is. By marking normal and mutant cells amongst their neighbours, and following their development, we can determine the cellular movements and interactions that underlie normal development and disease. These tools are required to assess the behaviours of stem cells and to be able to devise ways to manipulate them for therapeutic purposes to facilitate regeneration.

The availability of mouse models that are genetically well characterized and the wide range of genetic manipulations that can be performed make mice ideal for studying the genetic basis of brain development and postnatal stem cell biology. Since the DNA sequence of the mouse genome is available, any region of the genome can be experimentally manipulated in any desired manner.

Moreover, the relative ease of housing and maintaining a large number of mice compared to other mammalian species makes the mouse the animal of choice for the described studies.

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

In vitro (cell and tissue culture) models of mammalian stem cells that allow cells to be cultured in self- renewal or differentiating conditions have been a gold standard of stem cell research. Furthermore, current brain organoid models are good alternatives to animal models since they recapitulate cell-to- cell interactions better.

Why were they not suitable?

Although cell culture models are very powerful, it is not possible to study the full spectrum of developmental and regenerative signalling, and injury response in such systems because they lack critical microenvironmental components and the tissue dynamics that drive development and repair. Importantly, faithful multicellular tissue culture systems that recapitulate neonatal cerebellum development are not available. Our research, in addition to accomplishing our aims described here, will also provide a springboard to establish faithful cerebellar in vitro models for future use of the scientific community and may help replacement efforts in the long run.

Reduction

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

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

Experimental animals utilised in the procedures (Sum of all protocols except for protocols 6-8):

- 3800 animals

The number of animals is estimated using power calculations or based on previous studies that utilized similar techniques to identify the necessary number of animals to achieve the aims of this licence. Per the experimental paradigm, we anticipate using ~5-10 animals/condition. Details of power calculations are provided under each protocol.

Number of animals required for the generation of lines and continues line breeding (protocols 6-8):

- 700 animals

Considerations for general breeding for maintaining colony and generating experimental breeding pairs:

We plan to have about ~10 lines at a time. For each line, the breeding pairs (1-3) will be continuously bred to produce ~12 litters/year. We also will keep one stock cage of males and females each. Some of these animals will be used to establish primary cell cultures to reduce the number of animals needed to achieve the goals 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 have utilized online tools (NC3R's experimental design assistant, PREPARE guidelines) and performed power analysis to assess the minimum number of animals required to fulfil the objectives set in the licence.

Furthermore, when possible, we will generate primary cell lines from mouse brains which will allow us to test some of our hypotheses in vitro prior to performing elaborate experiments that may require the generation of new transgenic animals, therefore reducing the number of animals needed to fulfil the objectives we set up in this license.

Finally, we also carefully designed our experiments to obtain the maximum amount of data to be generated from each animal killed and hence reduce the total number of animals needed to achieve our goals.

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 studies and in vitro tests to allow validation prior to moving to large scale studies.

We will share tissue with neighbouring labs from our studies that we do not anticipate using.

We will optimize our breeding scheme to allow maximum usage of the animals and allow us to provide correct littermate controls.

Refinement

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

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

We will use the newborn mouse as a model to study how the brain develops, and the repair mechanisms after injury to the brain. Our research involves identifying and isolating brain stem cells, following their behaviours through development and in adulthood or after injury to the brain with the goal to understand how we can facilitate repair in the human brain. Our research involves genetically engineered mice to allow the labelling and manipulation of stem cells in the animals, and several different injury models that are carefully designed to mimic devastating human disorders that cause neural loss.

The mouse brain is a great model for studying complex stem cell behaviours at different stages of life and in disease. Such responses cannot be studied in other organisms or cell, or tissue culture models as previously mentioned. Over the years we carefully optimised our injury methods and experimental paradigms to cause the least amount of pain and distress to the animals, compared to some other previously published work that leads to robust injuries to the mammalian brain.

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

The efficient repair processes (regeneration) are observed in newborn mouse brains and the impaired repair phenomenon is a disease of the mature brain, therefore we cannot perform studies at a more immature life stage.

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

Handling: Animals will be handled minimally following the best practice guidelines to exhibit the minimum stress possible.

Food and drink: Some of our experiments may require Tamoxifen or Doxycycline given in the drinking water or chow. We closely monitor weight and food intake to ensure animals are feeding properly.

Solitude and companions: Unless otherwise needed (such as fighting) animals will be housed in groups to ensure social interaction.

Play: Our cages will be enriched to ensure play time for the animals.

Post-surgical care: After surgery, animals will be closely monitored. Proper anaesthesia and analgesic cocktails will be used as described in the protocols and these will be regularly revised in collaboration with the veterinary staff.

We have previously optimized the stated procedures to induce minimal pain and discomfort to the animals, but in the unlikely event that we observe an increased frequency of unanticipated signs of pain and distress, we will stop our studies and evaluate the procedure in question.

Importantly, some of our protocols involve the use of neonatal (newborn) animals. Assessing distress and welfare in pups could be more difficult. We will closely monitor feeding behaviour (such as the appearance of white milk in the belly and weight gain patterns) as well as whether the dams are taking care of the pups or whether any rejection after the procedure. In case of rejection, foster dams will be utilized.

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

We have and will continue to follow the PREPARE and ARRIVE guidelines. Additionally, we will closely follow resources such as the Breeding and colony management for genetically altered mouse guidelines by NC3R and humane endpoints in laboratory animal experimentation by 3R-Centre Utrecht Life Sciences.

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

We will perform monthly literature searches to assess whether there is any new knowledge in the field that will allow us to modify our plans and improve our research with respect to the 3Rs.

We will analyse our data and assess our outcomes regularly and frequently (at least monthly) to make sure that our proposed objectives are still accomplishable or if we need to revise our experimental plan/abort studies.

We will follow the NC3R e-newsletter for the latest developments and implement the relevant updates immediately.

37. Investigating the regulation of lymphocyte activation and function

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Immunology, Immunotherapy, Tolerance, Vaccine responses, Lymphocytes

Animal types	Life stages
Mice	embryo, 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?

To understand how T and B cells sense and respond to their environments in the contexts of health and immunisation.

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

Why is it important to undertake this work?

T and B cells are essential immune cells that function to protect individuals from infections by bacteria and viruses. They work together to promote immune responses that last over time (called memory) meaning that after exposure to a germ your immune system is better and more equiped at responding to any potential future infection. Through understanding how T and B cells sense and communicate through receptors on their cell surface, we can better understand the fundamentals of immunity. This is particularly important for a wide range of situations that can affect human and animal health:

Understanding how T and B cells co-operate is critical for the design of effective vaccines

Understanding when and how T and B cells mount an immune response is critical to understanding and preventing autoimmune diseases.

Understanding the regulation of T and B cell responses is critical to identifying potential molecules that can be targeted with drugs to boost the function of the immune system, called immunotherapies.

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

We envisage that this work will lead to important publications in reputable open access journals in the immunology/life sciences field. These will provide fundamental knowledge into the workings of the immune system.

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

The project will deliver benefit to immunology and immunotherapy researchers in the short-term through the generation of new knowledge and understanding of important drugs that are used to manipulate immune responses. It will identify similarities and differences between different immune regulatory pathways improving our understanding of the immune system and how to manipulate it. It is likely to also improve our understanding of the requirements for effective T and B cell co-operation.

In the medium to long term, these findings could inform new immunotherapy regimes to go forward into early human testing. It could also inform about optimal dosing strategies for mounting effective T and B cell responses which may impact on long term vaccine strategies.

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

In order to maximise the outputs of this work we have collaborations that will enable sharing of expertise and optimisation of our experimental models. It will also maximise the chance of success of the project.

We will disseminate knowledge through publications, and for important data these may be released early into the public domain through non-peer reviewed pre-print servers such as bioRxiv.

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

Species and numbers of animals expected to be used

- Mice: 8500

Predicted harms

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

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

Mice are used in these studies because: (i) the main components of their immune system

are shared by humans; this is essential where immune responses as opposed to the function of individual genes is being studied. Other less sentient animals provide an inadequate model of the immune system; (ii) a wide range of wild type and genetically manipulated strains of defined genetic makeup are available;

(iii) an extensive breadth of knowledge already in place, enabling the best possible analysis of the cellular and molecular interactions occurring during immune responses. In addition, we are using unique T and B cell activation monitoring system, which has been established in transgenic mouse lines.

We will typically use adult mice for experiments, since by then the immune system is fully developed.

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

Animals will be bred to generate mice for use in experiments (including genetically altered mice where the genetic alterations are not expected to any particularly adverse impacts on welfare). Typically upon adult age, mice will be injected with substances that promote an immune response through the activation of T and/ or B cells. Typically this may be an injection under the skin, in the foot pad, into the blood stream or abdominal cavity. This allows the substances given to travel to glands called lymph nodes where T and B cells reside. In addition we may administer by injections drugs to alter the behaviour of immune responses in order to better understand lymphocyte behaviour. Some experiments will be up to several days long, involving several injections. Other experiments will proceed for several weeks to months to assess the longer term outcomes of immune responses.

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

For injections, mice will experience momentary pain, which is expected to be mild and short-lived. Some mild weight loss can be observed following immunisation, but this is uncommon and short lived. Some mice may have swelling and redness on the base of the foot for up to a week.

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

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

The expected severity of most animals in our immunisation protocols is expected to be moderate (60%), with limited side effects, similar maybe to what humans experience following vaccination. For shorter immunisation/ immunomodulation the severity is likely to be mild (40%) to reflect reduced number of procedures.

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

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you

have considered and why they cannot be used for this purpose.

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

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

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

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

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

Established T and B cell lines

Why were they not suitable?

Cell lines which can be grown in dishes in the lab can not capture the complex interactions required to mount immune responses. In particular cell cultures do not have the organ structures that glands like lymph nodes do, meaning they can not give any insight into the research questions posed.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 project requires the breeding and maintenance of mice. In order to maintain colonies not all mice are subjected to further experimental procedures. We will utilise many different strains of mice to explore immune responses in our protocols. The number is based on historical use based on our previous experience and aligns with the current numbers used on previous licences. It is also informed by published data using protocols established.

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

We have consulted with the NC3R's Experimental Design Assistant tool. This helps to design optimal experiments. Typically we aim to use what is known as a "randomised block design" which permits us to detect differences even when we introduce variables such as different sex and age. This approach controls for these differences by randomly

assigning them to control and treatment groups. In this way we can maximise use of mice bred for the licence.

By using pilot experiments we will be able to determine the minimum number of mice to be used - typically we have determined that 6-8 mice per group will allow us to detect a reasonable treatment effect in most circumstances. However these calculations will be continually updated and improved throughout the life of the project.

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

Pilot experiments

Small numbers of animals will be used in pilot experiments for each new type of study performed to assess variation. For most models in this project, optimal group sizes and variation are already well established on previous project licences.

Inbred strains and efficient breeding

The use of inbred strains (matched for age and gender) will reduce the amount of variation and we have efficient breeding protocols to ensure that we breed the appropriate number of mice to maintain the different mouse strains.

Multiple read-outs from same animal

Multiple read-outs taken post-mortem from experimental animals will further reduce the number of animals required. In addition we always seek to see if tissue can be shared amongst our research group to minimise the number of mice 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.

Immunotherapy model.

We use a state-of-the-art model that can assess rapidly the function of drugs that target brakes that are exerted on T cell responses. This model has been refined to be able to produced data within a few days, minimising the duration of adverse effects experienced by the animals.

Cell transfer and immunisation

Protocols have been optimised by past experience and pilot studies. Immunisation will be done with the smallest amount of antigen and with established adjuvantsthat are also used

for childhood vaccinations, and doses have been tested in preliminary studies. We therefore expect very few adverse effects.

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

This work has to be conducted in mammalian species, mice in our case, because the immune system in mammals is much more complex than that seen in invertebrates or other vertebrates. Hence, studying rudimentary immune systems will not lead to a significant increase in our understanding of the regulation of the immune system. The mouse is the species for which its immune system has been the most intensely investigated and for which there are the most reagents and information available. Embryos can not be used as the immune system has not developed and terminal anaesthesia is not an option due to duration of the experiments.

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

Monitoring of mice

Mice will be monitored particularly closely during the first 48 hours when adverse events are most likely, therefore if any issues arise these can be dealt with as soon as possible to minimise mouse distress and discomfort.

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

We will use refined handling techniques as published by NC3Rs on the handling and administration of substances and we regularly consult the NC3Rs website (www.nc3rs.org) and report results according to ARRIVE2.0

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

We will regularly consult the NC3Rs website (www.nc3rs.org) and new staff will be directed to these web pages. Where new 3R advances occur we shall work with staff in our facility to adapt protocols or techniques. We will also seek advice and latest information through our Named Information Officer (NIO).

38. Cellular and molecular characterisation of neuroimmune interactions in the context of pain

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Chronic pain, Neuroscience, Immunology

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?

Everyone knows someone who suffers chronic pain. It is an extremely common condition, and yet, we lack safe and effective medications to treat it. Our aim is to advance our understanding of what happens in the body when we experience chronic pain. In particular, we will focus on how immune and other tissue-resident cells talks to the nervous system and vice versa.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 team regularly interacts with people living with chronic pain conditions, who advise and inform on our work plans. Their experience tells us what epidemiological data also show very clearly: chronic pain majorly reduces your quality of life. For example, people told us that “It's painful everyday and depressing when I'm sleeping cause sometimes it becomes the cause of me waking up”. Some also just urged us to “Please just find a cure”. We hope that our research - which bridges an interdisciplinary gap between neuroscience

and immunology - can bring us one step closer towards this goal.

What outputs do you think you will see at the end of this project?

We hope that our work will eventually lead to safer and more effective pain medication for patients. It is an approach that has worked in the past. Over the last 20 years, pain research in animals has given major breakthroughs in terms of medication. One is already in the clinic (biologic treatments for rheumatoid arthritis), while others are still being developed, e.g. targeting particular pathways around the inhibition of growth factors, like NGF.

Our work will also contribute towards better understanding of the communication between sensory nerves and other cell types in their environment. We will publish our results open access and on pre- print servers, alongside searchable raw data whenever feasible.

Who or what will benefit from these outputs, and how?

In the short term, our work will benefit other scientists across a variety of research fields, including immunology, inflammation biology, stromal cell immunology and neuroscience. Besides publications, we produce a lot of datasets which others can use to inform their own research interests. We make them available online and ensure that they are easy to search and use.

In the longer term, i.e. beyond the life-time of this licence, our work is designed inform better analgesic drug development. We achieve this by working across disciplines with other field-specific experts and by collaborating with partners from the pharmaceutical industry.

How will you look to maximise the outputs of this work?

Our work is always very collaborative - with at least one other group involved, usually from the inflammation biology or immunology fields, while our group provides input on neuroscience and sensory neuron biology. This ensures that our results will be disseminated across several independent scientific communities via conferences and publications.

My group is also very keen on the publication of null data, which we make a special effort to make available to the community, e.g. on the Wellcome Open Collection. We also use pre-registration platforms when appropriate.

Finally, we make sure that our datasets are easily accessible online, either by sharing them through our own purpose-built webtools (http://rna-seq-browser.herokuapp.com/, avg. 140 users a month in 2022) or by using those of others (e.g. the Broad Institute Sequencing Browser).

Species and numbers of animals expected to be used

- Mice: 16500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures,

including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are a good model system to study the communication between nerves and other cell types. They can be manipulated genetically, which allows us to mark specific cell populations or knock-out particular proteins. Their sensory and immune systems are also very well described, and our work can therefore build on a solid foundation of prior scientific knowledge.

We need to use mice across the entire life span, since we are interested both in how early life experiences can influence later pain perception and in how pain (an age-related condition) develops later in life.

Typically, what will be done to an animal used in your project?

The majority of our mice are simply being bred as transgenic mice and will not be used for any experimental procedures. Breeding is classified as “mild”, since the animals do not experience any undue suffering, apart from being held in an animal facility rather than being allowed to roam free. We will ensure that breeding will be carried out efficiently, with the production of surplus animals minimised and animals not kept alive unless needed for future experiments or future cohort generation.

Sadly, since we are trying to model pain experienced by patients, some of our mice will have to undergo a procedure that causes them temporary pain. For instance, we use well-established models of arthritis, where the mice develop inflamed knee joints, just like a patient would. We then try and understand this condition & test new treatments. Any pain experienced by the animals will be limited in both duration and intensity, e.g. in the case of arthritis, a mouse will have sore joints, but otherwise still explore and interact with other mice – the equivalent of a patient being in discomfort but still being able to work on the day. At the end of an experiment, the animal will be put to sleep. We will look at their nervous system after death to figure out what has been happening in their body.

What are the expected impacts and/or adverse effects for the animals during your project?

Most of our animals will not experience many adverse effects, as they will simply be bred as 'transgenic' animals. For those mice, the main adverse effects will be slight pain and distress, lasting 10-30 seconds, while taking a tissue sample to determine their transgene status. Sometimes, when required, animals will experience whatever adverse effects come with aging naturally, e.g. they risk developing tumours or some other age-related disease. Obviously, as soon as these are discovered, the animals will not be kept.

For those animals who are used to model pain conditions, e.g. nerve injury or arthritis, they will experience pain as their main intended adverse effect. However, we have very strict end-points in place that ensure that their pain does not exceed a certain level. For example, our animals will continue to gain weight, and groom and interact with their cage-mates normally. For the most part, our mice will stay in an experimental model for a few weeks.

Sometimes, our animals will undergo brief surgeries, either to induce a particular pain model or to manipulate their genetics. In that case, the adverse effects associated with

surgery, such as temporary pain, will be treated with analgesics. The negative effects from the surgery should not last more than 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)?

Projecting forward from our historical data, we expect around:

50% of mice to be used just for breeding (subthreshold category);

14% of mice to just experience a mild procedure (e.g. a s.c. injection to induce transgene expression followed by tissue harvest via perfusion fixation);

36% of mice to be within a moderate category protocol where they experience surgery or where pain is the main intended adverse effect.

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 gaps we have in our understanding of pain are related to highly specific processes that take place in nerve cells. To improve our knowledge we need access to these nerve cells. Naturally, human donation is not possible in this case.

Additionally, pain experienced by patients is a complex, multidimensional experience that is influenced by extraneous factors like motivation and emotional state. It is therefore impossible to model fully in cultured cells or non-protected animal species.

For example, while we can model some molecular interactions between immune cells and stem-cell derived sensory neurons, we cannot model the whole-body experience of pain that is the functional consequence of these interactions.

Moreover, even certain molecular interactions we unfortunately cannot model without animals, as stem-cell derived sensory neurons cannot be produced in all the different phenotypes that exist in vivo.

Finally, non-protected animal species have very different immune systems from humans, meaning that many neuro-immune interactions we need to study cannot be modelled correctly.

Which non-animal alternatives did you consider for use in this project?

We considered organoid and stem-cell models, and are using them whenever we can (i.e. when the organoids and/or stem-cell derived neurons have a phenotype that resembles that we want to study in humans).

Moreover, our work usually starts with data mining, e.g. of RNA-seq datasets, which ensures that we do not unnecessarily repeat work the results of which are already publicly available. We also use ligand-receptor binding analysis to ascertain that the right molecular machinery is at least present in principle on the cell types that we are studying

We have also considered mathematical and computational models, as well as epidemiology.

Why were they not suitable?

Unfortunately stem cell-derived sensory neurons do not recapitulate the full phenotype of 'original' human sensory neurons. For example, right now, we cannot make a type of sensory neuron that is responsible for us perceiving pain but that can also interact with immune cells (a so-called 'CGRP+ sensory neuron').

Mathematical and computational models are not suitable as they not provide information on whole- body pain phenotypes.

Finally, epidemiological studies lack the ability to make a causal connection between a particular neuro-immune interaction and pain, which is what we are hoping to identify with our 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?

For individual experiments, we think about the minimum difference between groups that we would be interested in, e.g. we want a drug to reduce pain by at least 2 points on a given scale. There are statistical methods that then help you determine the number of animals you need for a given experiment given your minimum difference of interest.

For the licence as a whole, we know how many animals we have used over the past five years. We know that we will use a similar number going forward, since we have a similar number of grants and personnel going forward over the next 5 years.

Obviously, as we develop new alternative approaches, e.g. as our stem cell models improve, we may find that we need to use fewer animals than we are planning at present.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We always make sure that we conduct statistically and scientifically rigorous experiments. The design of each experiment is discussed by at least two people (myself and the team member who will carry out the work). However, frequently, we additionally discuss particular designs as a group in internal and external lab meetings (e.g. with collaborators).

My staff are trained in the use of design frameworks, e.g. right now we follow the recently published EQIPD framework (https://www.nature.com/articles/s41592-022-01615-y), and of course always use ARRIVE 2.0 guidelines for reporting.
For example, we use randomisation and blinding, and power calculations to ensure that we use the right number of animals for each question at hand. We plan within-animal designs where possible to increase our power, and we carefully think about which statistical test will give us the maximum power for the analysis of a given dataset.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Our work usually starts with data mining, e.g. of RNA-seq datasets, which ensures that we do not unnecessarily repeat work the results of which are already publicly available. We use ligand-receptor binding analysis to ascertain that the right molecular machinery is at least present in principle on the cell types that we are studying.

Where feasible, we use historical tissue that we have collected from prior studies or rely on tissue from collaborators. We are also sharing tissue that we have generated with collaborators (e.g. PhD students whose projects I'm familiar with through a training programme that I lead).

Finally, we make sure that we manage our colonies effectively to avoid unnecessary breeding.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use models of arthritis, neuropathy and inflammatory pain. For every experiment, we ensure that we choose the model that causes the least pain and suffering to the animals, while still being scientifically appropriate. Specifically, we will make sure that mice only remain in a pain model for as long as scientifically necessary. We will choose acute inflammatory insults over chronic ones when possible. When possible, we conduct short-term experiments under terminal anaesthesia, e.g. to assess the effect of an inflammatory mediator on neuron function using optical imaging. Finally, where possible, we will use an arthritis model that causes pain in only one joint. Only when we are certain that a particular treatment avenue is very promising would we then move on to a more painful, but also more clinically relevant model that can cause pain in multiple joints – something that is

usually seen in patients.

Why can’t you use animals that are less sentient?

We need to determine whether our treatments and interventions affect whether an organism experiences pain. While pain can be modelled e.g. in worms and drosophila, these species have very different immune systems to people (e.g. they lack a so-called 'adaptive' immune system). Since we are interested in neuro-immune communication, these models are too reductionist for us.

Moreover, it is difficult to model the emotional and affective components of pain, as well as spontaneous pain in less sentient species. As in, pain in worms is usually measured by measuring how they react to being poked. However, chronic pain in people is usually not evoked. It is spontaneous, and the emotional mindset is very important to the ultimate pain experience. In mice, we can begin to study these things, e.g. using behavioural setups that give them the choice to seek pain relief or that capture the way they move or interact with each other.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We are looking to implement the use of a new behavioural setup that would allow us to use video technology to assess various spontaneous pain behaviours in a stress-free, darkened cage. We are already using home cage monitoring, which also means that we can minimise handling-induced stress, but it is unfortunately not sensitive enough for many of our models, as the pain they induce is purposely mild.

In general, animals that have to undergo surgery or pain models are carefully monitored for their pain levels and general health (see protocols for detail).

For surgery, we provide peri- and post-operative analgesia, as well as enrichment, e.g. in the form of peanut M&Ms that can double as food-supplements, but are also used by the mice for species-typical behaviours (i.e. they like to bury them in their bedding).

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will be using the EQIPD guidelines for our experimental design (https://www.nature.com/articles/s41592-022-01615-y) and the ARRIVE 2.0 guidelines for reporting.

We are also relying on:

Guidelines for refining administration of substances:
https://journals.sagepub.com/doi/10.1258/0023677011911345

The Laboratory Animal Science Association Guidelines for Aseptic surgery:
https://www.lasa.co.uk/PDF/LASA_Guiding_Principles_Aseptic_Surgery_2010.2.pdf

Refinements for the use of mice in arthritis research:
https://link.springer.com/article/10.1007/s10787-015-0241-4

Guidelines for welfare assessments:
https://journals.sagepub.com/doi/10.1258/la.2010.010031

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

My team and I regularly access the NC3R website to see their latest news and check on their resource library.

We also keep abreast of any new NC3R-relevant technologies through collaborators and/or scientists at conferences and through publications. For example, we keep up to date on news regarding better humanised cell-culture models and refinements in behavioural tests. Just now, we are testing an improved protocol for the differentiation of sensory neurons that can supposedly generate a wider variety of sensory neuron-subtypes. We are trying this protocol even ahead of its full publication, since it has been made available as a pre-print on bioRxiv.

We will discuss new advances in our team meetings and seek training where necessary before implementing them.

39. Investigating the use of aspirin as a chemopreventative strategy in ageing mice

Project duration

0 years 6 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Cancer, Ageing, Aspirin, Mitochondria

Animal types	Life stages
Mice	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 work carried out under this license aims to understand the influence of age-related changes in the efficiency of cellular energy production on the use of aspirin as an intestinal cancer prevention strategy.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Colorectal cancer is the third most commonly diagnosed cancer in men and women, with up to 42000 cases diagnosed in the UK each year, resulting in 16000 deaths per year. This is because cancers are often only detected when they are very advanced and the

more advanced cancers often respond poorly to treatment. To develop better treatments which are likely to have a positive effect in patients, and more importantly, preventative strategies, we need to better understand a number of features of how cancers behave. These include understanding how they form, how they grow and they progress to more dangerous disease. 60% of human colorectal cancers have dysfunctional mitochondria. Mitochondria are tiny energy generators that exist in large numbers (100s-1000s) inside human cells. Mitochondrial dysfunction in these tumours is caused by alterations in a small amount of DNA inside the mitochondria themselves, known as mitochondrial DNA. These can change the behaviour of the cancer cells and make them grow faster and resist cell death in the early stages of the disease.

Aspirin has been shown to be a promising intestinal cancer preventative agent. The mechanisms underlying the benefits of aspirin administration are not fully understood, but it is most likely due to changes in the function of the immune system and causing cancer cell death. As we have shown that mitochondrial dysfunction causes resistance to cell death, this may alter the cellular response to aspirin treatment. To understand this, we will induce tumours in mice that we have genetically engineered to have mitochondrial DNA mutations and test the effect of aspirin therapy on both the tumours and the normal intestinal cells. We aim to identify whether the cells with mitochondrial dysfunction respond differently to aspirin and investigate the molecular mechanisms that cause these responses.

What outputs do you think you will see at the end of this project?

Work conducted under this project license will generate new data and knowledge about how mitochondrial dysfunction affects intestinal cancer development. We aim to understand how mitochondrial dysfunction affects the cancer cells and the molecules within them and test their sensitivity to aspirin as a cancer preventative strategy.

Our work will be presented at scientific meetings and published in scientific journals to share the knowledge that we gain with the wider scientific community. This will help to advance knowledge in the field for the ultimate benefit of patients.

Who or what will benefit from these outputs, and how?

In the short term, scientists in both academia and industry will benefit from the discoveries generated under this programme of work. This could be due to the development of new research tools, experimental approaches, or identification of new pathways which, when targeted, yield therapeutic benefit.

The long-term aim is to benefit patients through the development of new prevention and treatment strategies.

How will you look to maximise the outputs of this work?

By presenting our discoveries at national and international scientific meetings, publishing our research discoveries, and through collaborations with academics or the pharmaceutical industry, we will be able to maximise the impact of knowledge gained under this programme of work.

Wherever possible we collaborate with others to share tissue samples, cell or mouse lines, and provide training in methods through collaborative research or participation in

workshops.

Species and numbers of animals expected to be used

- Mice: 10

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 humans, age is the biggest risk factor for colorectal cancer development, therefore aged mice will be used.

To study disease biology and ask how a protein effects the disease process we need to use genetically modified mice so that they either do not express that protein or express a modified (or mutant) form of the protein. The proteins we will modify have been identified in human genetic studies to increase the risk of developing intestinal cancer and mitochondrial dysfunction. We can genetically modify the mouse genome to generate mammalian models in which to study mitochondrial dysfunction in intestinal cancer. We need to do these experiments in whole animals, as during cancer development there are complex interactions between the tumour cells and the cells in the surrounding tissues. There are currently no human or mouse cell models (*in vitro* models) capable of a comprehensive study of mitochondrial dysfunction in cancer development in interacting physiological systems. We cannot use less sentient animals such as insects or fish as they lack some of the immune cells present in humans, meaning that they cannot model the cancer development and the response to aspirin treatment accurately. This is essential to support the translation of our research into humans.

Typically, what will be done to an animal used in your project?

As age is the biggest risk factor for colorectal cancer development, we will age the mice before we begin the treatment or induce the tumours to accurately model the aged tumour microenvironment (100% of mice)

In our genetic model of intestinal cancer, the tumours will need to be induced by injection of a chemical (tamoxifen) into the mouse's abdomen. The cancers will then develop over a number of weeks (~50% of mice)

Mice will be administered with oral aspirin (or the solution which the aspirin is dissolved in as as control) to see how it affects normal intestinal cells and whether it is able to reduce tumour growth. (100%).
We will take urine and faeces samples to assess, how the body metabolises the aspirin. (100%).

At the end of the experiment all mice will be humanely killed and their tissues harvested and examined.

What are the expected impacts and/or adverse effects for the animals during your project?

In our mouse models, mice may show signs of sickness e.g. hunched posture, diarrhoea, ruffled fur, or look pale. We will closely monitor all of the mice using a score sheet and give supportive care if required. Any mouse which reaches our pre-defined endpoints e.g. if it loses 20% of it's body weight, will be humanely killed.

As age is the biggest risk factor for colorectal cancer development, we will be inducing tumours in aged animals. The mice may develop age-related effects such as; weight loss, greying of hair or hair loss, ruffled fur, ocular deterioration (eye cloudiness or cataract formation) reduced activity, reduced subcutaneous fat and signs of kyphosis (curving of the spine). We will closely monitor all of the mice using a score sheet and give supportive care if required. Any mouse which reaches our pre-defined endpoints e.g. if it loses 20% of it's body weight, will be humanely killed.

Intestinal cancer models will develop tumours over a number of weeks, predominantly in the intestine. As the tumours get bigger, the mice may show other symptoms, predominantly weight loss and anaemia. In our extensive experience with the genetic models used in this study, these symptoms typically begin to present 20-25 days after tumour induction. In this study, the mice will be killed 23 days after tumour induction, therefore the mice are unlikely to experience these effects for more than 2-3 days. Mice will be monitored daily from the point at which they start to develop tumours using a clinical score sheet. Any mouse which exhibits clinical signs indicating that their condition is deteriorating e.g. more than 20% weight loss or severe anaemia 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)?

Only mice will be used in this program of work.

We expect ~100% of the mice will experience moderate severity either because they will develop tumours, or they will experience a combination of procedures such as genetic alteration combined with administration of therapy and the effects of normal ageing.

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 is a complex disease, which also involves multiple cell types. For example, the way a tumour grows can be affected by the numbers and types of immune cells, which are present in the place in the body in which it is growing. We wish to understand how mitochondrial dysfunction influences growth of the tumour and it’s response to aspirin. It is only possible to study this in the whole animal due to the interplay with the immune system. We also want to test the effectiveness of aspirin to prevent or slow cancer

development. It is important that this is done in live animals to test the effects of medicines on the whole body. This is both in terms of how the body takes in the medicine, how the medicine moves around the body and how the body get rids of the medicine (pharmacokinetics), and what the medicine does to the cells (pharmacodynamics). This cannot be predicted in human tissue/cells or cell culture systems because of the complex nature of the way the body's systems work together.

Which non-animal alternatives did you consider for use in this project?

Wherever possible, we will use human tissue/cells or cell culture systems to replace animal models of mitochondrial dysfunction in cancer. We have accumulated archival tissue banks of preserved tissues from our previous models and from healthy and diseased human tissue. These samples are used in multiple ongoing projects to minimise the number of animal models used.

We routinely use samples of human cells to understand and model the biological processes affected by mitochondrial dysfunction both in normal and cancerous cells and to perform initial drug testing.

We have started developing methods to grow 'mini-guts' or small tumours in plastic dishes in the laboratory as an alternative method for drug screening and to understand the effect of mitochondrial dysfunction on cancer cells. These advances will help to minimise animal use.

Why were they not suitable?

Whilst these are useful tools, there are limitations of cell cultures systems, these include;

Cells grown in culture need a plentiful supply of nutrients, particularly glucose. This means that they are often not reliant on normal mitochondria to function and therefore when they have mitochondrial dysfunction they do not behave in the same way that they do in the body. These abnormal behaviours could lead to the identification of non-relevant pathways or fail to predict drugs that are likely to be ineffective in the disease.

Cells grown in petri-dishes sit on plastic, which changes their biological characteristics and they become "super sensitive" or fail to recapitulate their normal biological role.

The in vitro screen and systems we use prior to animal studies are extremely useful for aiding our basic knowledge and for initial drug screening, and we use these systems to reduce animal use however, they cannot fully model the behaviour of a drug in a complex biological system

Whole-body work is required to understand cancer pathology, as there is evidence for the interplay of between different tissue/organ systems. As organoid systems become more advanced, this may become possible, but technology is currently lacking for these applications.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise

numbers consistent with scientific objectives, if any. These 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 this license we are completing a study that was started under the previous license. Therefore we only need a small number of mice to ensure that we have enough mice to generate the data required. In order to calculate the numbers required for the whole study (this license combined with the previous one) we used knowledge from previous studies to mathematically calculate the minimum number of animals needed in each group to generate data, which allows us to answer our scientific questions. By doing this we can minimise use but be confident that the differences in a scientific measurement between two groups is meaningful and has not been obtained by chance. We have also consulted with our institutional statisticians whilst calculating the group sizes we require.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The PREPARE guidelines were and will be, referenced during the planning phase of experiments; this includes thorough examination of the literature, use of pilot studies, prevention of observer bias (by using standard operating procedures), ensuring sufficient staffing and competency is in place to conduct the selected tests, and deciding on which characteristics of the animal are essential to each specific study. Standard Operating Procedures have been written and used routinely for previous projects. This standardises the way the data and metadata is collected and reduces the variability and therefore the sample size.

In addition, constant evaluation of procedures and refinements will minimise the number of animals used in this project.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Statistical analysis is performed to determine the minimum numbers of animals needed to generate biologically meaningful data.

Tissue is regularly shared between projects to maximise outputs from animal procedures and minimise numbers of animals used.

Wherever possible, we will use human tissue/cells or cell culture systems to replace animal models of mitochondrial dysfunction in cancer. The group have accumulated archival tissue banks of preserved tissues from our previous models and these samples are used in multiple on-going projects to minimise the number of animal models used.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We have chosen this disease model as it is the lowest severity model that can be used to answer our research questions. To understand the role of mitochondrial dysfunction in cancer we need to use genetically altered mice in which the mitochondrial do not work properly. We will monitor the behaviour of these mice to ensure minimal suffering.

Most of these mice will develop tumours of the intestine, which we will induce via injection into the abdomen. The model of cancer we will use is very well-studied, has a predictable course of disease and we are very experienced in monitoring the clinical signs of tumour development to ensure that the study is ended before the animals are experiencing unnecessary suffering or distress.

Why can't you use animals that are less sentient?

The mouse is the lowest mammalian species in which we can perform the full range of genetic manipulations that we need to understand how mitochondrial dysfunction affects intestinal cancer development and response to treatment. It is possible to carry out some manipulations in frogs and fish but the processes being studied here are being studied in a mammalian context and, although other animal and non-animal species can be informative in this regard, they cannot replace studies specifically in mammals. Mice remain the model of choice due to the volume of genetic information available, their relevance to humans and the relative ease of generating, establishing and preserving mouse colonies. It is well accepted that cancer has an inflammatory component. Fish and insects (e.g. flies) lack the same broad range of immune cells found in humans, which means that there are differences between fish and mammals that could affect the disease biology. Some genes are not conserved between these species and mammals therefore some of the disease mechanisms may not be the same. Therefore, drugs that target those cells or disease pathways may not work in these systems due to fundamental differences in the biology of the species compared to humans.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All animals are checked regularly and supportive care is readily provided to minimise distress or suffering and improve animal welfare.

We have also actively engaged in a program of low-stress handling which has shown that tube handling can reduce stress in experimental models. We have used this method in our studies for some time now and have adopted it as standard practice.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

For all models and optional procedures good practice guides will be used to help refine the model as described in the ARRIVE guidelines, Laboratory animals special article 2015, 49 (s1) and the NC3Rs guidelines.

Workman et al in 2010. British Journal of Cancer (2010) 102, 1555–1577 NCRI guidelines will be used to perform and monitor cancer studies.

We will also regularly consult the following sources to ensure the highest standards of animal welfare using the most refined approaches:

The UK Home Office

The European Union

Code of Practice for Housing and Care of Animals Bred, Supplied or used for scientific purposes

RSPCA Animals in Science guidelines

UFAW Guidelines and Publications

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

There are many sources in which provide information regarding 3Rs advances, these include; the NC3Rs website, NC3Rs seminars/events and emails, scientific publications and published guidelines as well as continued professional development e.g. local seminars, regular communication with the NACWO, NIO and veterinary team and academic collaboration with the welfare group.

As information on welfare or technical improvements, alternative less severe models or new non- animal model systems become available, an appropriate strategy within the research group and veterinary teams will be implemented to ensure that animal use and suffering is minimised. This will include testing new models (animal or non-animal) and modifying procedures.

40. Information processing in neural circuits

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Neural circuits, Sensory processing, Mental illness, Novel therapies, Optical methods

Animal Types	Life stages
Mice	Pregnant, adult, juvenile, neonate, embryo, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We aim to find out how electrical activity in networks of nervous cells in the brain enables the representation, processing, and storage of sensory information in the brain.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Improving our understanding of how the brain processes information coming from our senses is in the first instance a matter of considerable, fundamental scientific interest. In the longer term, the insights gained from this project should also allow us to identify new therapeutic targets for mental illness that affects sensory processing (e.g., schizophrenia or ADHD) and storage of sensory information into memory (e.g., Alzheimer's disease and dementia).

What outputs do you think you will see at the end of this project?

The project will generate output in the form of peer-reviewed research findings; non-specialist public communications; leads for novel treatment strategies for mental illness.

Who or what will benefit from these outputs, and how?

The main beneficiaries will be other researchers studying how the brain processes information from our senses in health and disease. Through the development of potential new treatment strategies, researchers in industry and ultimately the general public stand to benefit from the outputs of this project.

How will you look to maximise the outputs of this work?

We have been very successful in the past to maximise outputs and their impact through varied multi- and inter-disciplinary collaborations. These collaborations typically produce benefits and outputs bigger than the sum of their individual contributions.

Species and numbers of animals expected to be used

- Mice: 8500 (some animals may be purchased-in instead of bred under authority of the license)

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 brains of mice process information from their senses, such as touch, taste or smell, very much like in humans. The use of mouse models for behavioural testing has been refined over many decades.

Additionally, mouse models allow us to use very specific, targeted genetic modifications. These genetic modifications allow, for example, to manipulate brain activity in a very precise manner or model human neuropsychiatric disease through the transfer of human disease risk genes to the mice. To map connections between brain areas, we will require the use of early life stages. All behavioural experiments will be carried out in adult animals.

Typically, what will be done to an animal used in your project?

Part of the project will involve the generation of genetically altered mice to allow us to investigate the functions of particular molecules and cell-types in sensory processing. These animals are expected to be not fundamentally different in the way they behave from wild-type controls and thus expected levels of severity will be mild. In about 5% of the animals, we will need to trim some of the whiskers of the animal to be able to relate neural signals to touch sensation in specific whiskers. This has minimal effect on animal well-being. In about 30% of the animals it will be necessary to inject substances into the brain to deduce anatomical structures and function or implant electrodes or small windows through which brain activity can be viewed with a microscope. This will be carried out under general anaesthesia typically lasting no longer than 90 minutes, in sterile conditions,

with about 5% of the animals being humanely killed before regaining consciousness (e.g., where tracer substances require very short incubation time) and the other animals being recovered with appropriate post-operative care and only causing moderate amounts of discomfort to the animals in the study. The behavioural tasks we will use to record conscious, sensory perceptions such as the mouse touching an object with their whiskers, are painless. In some cases, it will be necessary to motivate the animals to perform these tasks by rationing their food or water during testing. This may result in a small, temporary weight loss, but this will always be monitored carefully, and extra food or water provided if this occurs. Rationing results in lean, motivated animals and will typically last about 14 weeks. The availability of modern techniques for monitoring or altering neural activity in particular regions of the brain make it possible to carry out all of this work in a manner that should cause only moderate amounts of discomfort to the animals in the study. For example, surgical operations for implantation of ultrafine microelectrodes or for inserting genes into the brain will be carried out under general anaesthesia, in sterile conditions, and with appropriate post-operative care. The adverse effects that may occur following surgery include transient pain and bleeding, but their incidence is likely to be less than 5% of the animals undergoing surgery. Chronic implants for recording neural activity or for delivering flashes of light for the purpose of altering that activity are small and light-weight, and do not materially affect the animal's quality of life. A relatively small percentage of animals (about 12%) will be used in tests where the head needs to be fixed to enable stable recordings of brain activity. These recordings typically last about 45 minutes per day and are repeated daily for 2-3 weeks. In these tests, the animal is supported on a moveable platform that allows the animal to perform behavioural tasks, for example, navigating through a virtual maze projected onto screens. This method is now very established and well tolerated by mice displaying the same behaviour as when walking freely and should cause only mild amounts of discomfort to the animals in the study. Animals will be killed humanely at the end of the experiment.

What are the expected impacts and/or adverse effects for the animals during your project?

Some animals will experience transient, post-operative pain, typically lasting no longer than 1-3 days during which pain relief will be provided. Some animals will be fitted with chronic head implants (typically just a small, light-weight and thin, implanted window about 3 mm in diameter, in the skull) for typically about three months or until the scientific end is reached. In our experience, these implants do not noticeably affect animal welfare or behaviour.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The majority of animals (>50%) will be used for breeding and experience sub-threshold severities. Of those animals used in procedures beyond breeding, about 5-10% will experience Mild severity and about 30-35% 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?

Our project investigates how the brain processes information from the senses. Currently, this can only be studied by using the brains of animals or humans, as our understanding of brain function is too rudimentary to generate realistic mathematical models for testing. Brain imaging techniques available for use in humans such as fMRI, lack the sensitivity to observe changes in the properties of individual, identified brain cells in response to sensory stimuli. Moreover, a key aim of this project is to try manipulating brain activity in a very controlled, targeted fashion using genetic modifications to specific brain cells, which is not available in humans. Additionally, we aim to relate brain cell activity to the underlying neural circuitry at a microscopic level. This requires the use of post-mortem tracing of neural connections using staining methods, which would not be ethical or practical to carry out in humans.

Which non-animal alternatives did you consider for use in this project?

We considered human cell in vitro models and mathematical models of brain function.

Why were they not suitable?

Lower animals or human brain cell in vitro models do not nearly model human brain function in health and disease sufficiently as well as the mouse. Too little is known about brain function in general, to make mathematical models a suitable replacement given the project aims.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Yes. Calculations have been carried out to determine the necessary number of animals for each experiment, ensuring significance of our results but also minimising the number of animals used. We are additionally able to keep animal numbers to a minimum by using cutting edge methods, such as optical recordings of many hundreds of identified brain cells at a time, that yield large amounts of data and experimental designs that allow multiple measurements to be made from each animal.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We estimate effect sizes and adjust required numbers of animals to the minimum required to test our working hypotheses. Furthermore, we employ within-subject experimental designs wherever possible to reduce the number of animals required.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will work closely with our animal facility to ensure efficient breeding strategies. Additionally, we will use pilot studies when using new approaches to estimate effect sizes. We will also continue to make use of computer modelling of brain activity to reduce the conditions we need to be explore in experiments, thereby reducing 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.

Mice will be used because they are animals with a sensory system that is comparable to that in humans.

The impact on animal welfare will be minimised by carrying out procedures in state-of-the-art facilities and using best practice methods. Breeding and animal colony maintenance, including genetically altered mice, will follow the Home Office assessment framework for efficient breeding and maintenance. We will only use genetically altered mice that exhibit a mild phenotype (e.g., with no effects on feeding or welfare) or no measurable behavioural phenotype (e.g., mice producing a fluorescent marker in certain brain cells). Where animals undergo surgical operations, these will be carried out very carefully under anaesthesia and sterile conditions, and the animals are given painkillers and will be closely monitored until they have fully recovered.

Sometimes it will be necessary to regulate the food or water intake in mice in order to motivate them to perform behavioural tasks for a food or water reward. We have very strict guidelines in place to mitigate any harm from this food or water regulation, as well as for the behavioural tasks used.

The use of state-of-the-art methods will help reduce the impact on animal welfare while, at the same time, increasing the amount of scientific insight that can be obtained from each experiment. These methods include, for example, the introduction of genetic modifications to specific neurons, allowing us to change their activity in a precise fashion.

Animal welfare is regularly monitored. Clear instructions for all protocols ensure that procedures are aborted before exceeding set limits for impact on animal welfare.

The data obtained from these experiments will be used to refine computer models of the

brain that will help to guide subsequent experiments and contribute to a reduction in the number of animals needed.

Why can’t you use animals that are less sentient?

Our project aims to unravel the biological principles of sentience, so the basis for feeling and perceiving things using our sense, itself. Therefore, a minimum level of sentience is required in the animals used in the project. Furthermore, we hope to extrapolate our findings in mice to the brain function of humans, particularly in neuropsychiatric conditions, and therefore require animals with a minimally similar brain architecture.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We employ increased monitoring whenever there are possible welfare costs to the animals. For example, animals receive daily post-operative care, including pain relief where necessary, for at least one week after surgery. We also have strict, staged SOPs for habituating animals to handling staff and experimental apparatus, thereby minimising potentially stressful situations for the animal.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We regularly review our procedures and SOPs against the published literature, particularly peer- reviewed protocols (e.g., Nature Protocols) and use the published resources on the NC3Rs website.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We keep abreast about advances in the 3Rs through regular newsletters from our Home Office Liaison, NVS, and NACWO. Group members involved in the project also attend relevant workshops and online courses. Furthermore, 3Rs and license matters are reviewed and protocolled in a separate agenda item in our weekly labmeetings.

41. Early management to sustain life and minimise deterioration after traumatic injury

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Trauma, Haemorrhage, Shock, Pathophysiology

Animal types	Life stages
Pigs	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Trauma is characterised by complex injuries involving significant blood loss. In military and disaster medicine as well as very rural areas the journey to hospital may take several hours. Depending on the situation there may be limited resources and equipment to treat the casualty on their way to the hospital. The overall aim of the research is to develop new treatments and assessment methods to save lives and improve long-term 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?

In civilian and military trauma, blood loss (haemorrhage) is a leading contributor to death. Haemorrhage is normally associated with tissue injury, which changes (and complicates)

the response to blood loss. Amongst those that died in civilian practice and recent military conflicts, but whose injuries were potentially survivable, blood loss was a contributory factor in about 90% of cases. There is a clear need to develop new treatment strategies to save the lives of casualties who have lost a large amount of blood. 'Air ambulances' have helped save lives due to the rapid transport of casualties to hospitals. However there are many circumstances when air ambulances are not available, for example bad weather, unsuitable terrain or military conflict zones without air superiority. It will then take much longer to get the casualty to hospital. This will increase the challenges to the care providers to ensure the casualty survives. They need to treat the casualty for longer with restricted resources. This project will address these challenges and is relevant to any situation where the time to hospital is prolonged.

What outputs do you think you will see at the end of this project?

The principal output will be new information on the effectiveness, and limitations, of new resuscitation strategies for casualties who have suffered traumatic injury, especially if the time to get them to hospital is long. This information will contribute to how casualties are treated, as well as for medical planning (where to place medical assets and what assets are required for example). This is important in any scenario where there are environmental or logistical constraints.

The outputs will be reported initially in internal reports and submissions of papers for peer-reviewed publication in medical journals as appropriate.

Who or what will benefit from these outputs, and how?

There are two main beneficiaries: the injured patient and medical planners. Our clinical colleagues will use the information generated in these studies to develop new and refine existing treatments. We design the studies in close collaboration with key (influential) clinical colleagues to answer specific research questions that cannot be comprehensively addressed in clinical studies. It would be unethical to conduct these initial investigations in a clinical trials as the expected benefits of the treatments may not be realised. Additionally, harms to the patients are unknown. We need to obtain detailed physiological measurements as part of the evaluations. These would impact on the treatment of a human casualty and would be impractical in a pre-hospital setting. However once we know which treatment is beneficial and why, the output of these studies will be translated to influence clinical practice. Data from the studies will also provide the essential background information needed to plan a clinical trial if one is needed. The very circumstance this project addresses, i.e. evacuation from a difficult environment, is the very situation in which it is hardest to undertake a clinical trial. The output is relevant to the treatment of trauma in any environment with limited medical equipment and with long evacuation times.

The impact of the studies are often seen within and beyond the lifetime of the Project Licence since the work is designed to answer specific questions for medical planners and those engaged in treating trauma casualties. We have a record of reporting our findings on an iterative basis and plan to continue to do so.
The results will provide the evidence base for new pre-hospital treatments for trauma to be used in disaster, military and rural medicine. The benefits to the patient will include:

Improved survival.

An improvement in physiological, blood clotting and inflammatory state during the early in-

hospital phase that will allow greater flexibility in choice of surgical treatment.

Reduction in post-surgery complications that often result in patients needing longer periods of intensive care.
Reduced morbidity (degree of ‘sickness’), and improved recovery.

The benefit to health systems will be:

A reduction in hospital stay required by casualties and therefore a reduction in the burden of trauma to health systems.

Guidance for planners regarding the clinical resources needed to manage casualties.
Guidance when planning for medical treatment of victims of terrorist incidents.
Work conducted under previous Project Licences has supported clinical changes in military and civilian resuscitation practices.

How will you look to maximise the outputs of this work?

We have a large, cross-disciplinary study team conducting these studies, which helps maximise the information gained from them. We collaborate with colleagues in other establishments with expert knowledge and skills that complement our own, thereby reducing the number of studies that would otherwise need to be conducted were the cross-disciplinary approach not taken.

In addition we collaborate with others to supply data and/or post-mortem tissue taken from our studies to support studies conducted in other establishments, again reducing the number of animals needed to achieve their aims.

Finally, we use material from studies such as those proposed in this Project Licence to support other projects in our organisation that would otherwise require the use of additional animals. One example is the collection of nerve tissue post mortem from our current trauma studies. We are working with clinical colleagues to understand the effect of trauma and treatments on nerve pathology. We are able to do this without the use of additional animals since the nerve tissue would otherwise be considered as ‘waste material’ from a study designed for a different purpose. We plan to continue this approach in the studies proposed in this Project Licence.

It should be stressed that no animal under this Project Licence will be killed specifically to provide tissue for another Project Licence. The post-mortem material referred to above is always surplus material from studies specifically conducted for the purposes set out in this Project Licence.

Results of all of these studies are shared as described in the outputs section

Species and numbers of animals expected to be used

- Pigs: Up to 350 over 5 years (approximately 200 pigs for the trauma models and 150 pigs as donors to provide blood products)

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures,

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 pigs because their cardiovascular responses to blood loss, injury and resuscitation are representative of those seen in human patients. In addition, pigs are of sufficient size to allow the range of physiological measurements essential in this study, and have a large enough volume of blood that the repeated blood sampling that is part of the study has no detrimental effect of itself. The blood samples allow us to characterise many of the facets of the responses to injury and resuscitation that can be measured in human patients. Although there are other smaller, less sentient species that also have comparable cardiovascular response to injury they are simply too small to allow the range of physiological measurements and blood sampling that are essential for this study.

Typically, what will be done to an animal used in your project?

Each pig will be subject to one protocol under non-recovery anaesthesia. There are two types of protocol. The first type of protocol models trauma and resuscitation to understand the physiological responses and test new treatment strategies. The second type of protocol provides blood/blood products from a donor animal that are used in the trauma resuscitation (treatment) protocols.

Each pig will be transferred from their home pen to a specially adapted anaesthesia pen. The pig will be sedated in this area using a drug that is used in human patients as a pre-medication before anaesthesia. Once sedated the pig will be anaesthetised in the same area, then transferred to an operating suite for either a trauma protocol or blood donation. The pig will remain anaesthetised throughout the protocol and killed humanely at the end of the protocol with an overdose of anaesthetic, without recovery of consciousness (non-recovery anaesthesia).

Trauma protocols

Under anaesthesia the pig will be prepared so that we can record a range of variables. In some cases this involves placing probes on the skin surface (for example to measure heart rate), whilst in other cases a tube (called a cannula) is introduced into a blood vessel to record blood pressure, or a probe is placed around a blood vessel during surgery to measure blood flow within the vessel. Tubes (cannulae) may also be placed into other organs such as the bladder to allow urine flow to be measured. These tubes can also be used to withdraw body fluids such as blood and urine for subsequent analysis and measurement. After this preparation is complete, a controlled injury will be made and blood will be withdrawn to cause a substantial blood loss. There is then a phase of basic initial treatment that involves administration of a limited amount of fluid (a salt solution called saline) if blood pressure falls too low. This replicates the initial treatment of human patients. The combined events up to this point set the scene for comparing different potential treatments in the subsequent phase(s) of the protocol. These potential treatments include resuscitation[1] , for example to compare the use of different fluids or blood products. Alternatively, or additionally, other treatments for example using drugs or oxygen will be started (in different groups). Throughout the protocol there may be repeated physiological measurements (such as blood pressure and blood flow) and blood/tissue sampling. This may continue for up to 12 hours, during which time there may be continued blood loss to mimic particular clinical scenarios. At the end of this period an overdose of anaesthetic will be administered and the animal killed without recovery of

consciousness.

[1]Resuscitation is a term that is widely used in medicine, particularly in emergency medicine. In the context of this licence it is specifically used to indicate administration of fluid into the cardiovascular system to treat the deterioration that is occurring as a consequence of the initial blood loss and injury. Examples of this deterioration include low blood flow to organs and damage because of lack of oxygen being delivered, clotting problems and the initiation of widespread inflammation.

Blood donors

Under anaesthesia the pig will be prepared to allow recording of basic physiological variables, e.g. heart rate. A tube (called a cannula) will be introduced into a blood vessel to allow controlled withdrawal of blood. Blood will be collected at rates guided by those used by the blood transfusion service for human donors. The main difference between human blood donation and collection of blood from pigs in this protocol is the use of anaesthesia and the much larger total volume of blood collected from the pigs. In this protocol sequential units of blood will be collected until the collection rate is less than blood transfusion guidance, or we are unable to collect a complete unit. The rate of blood flow during collection is important because if the rate falls too low changes, such as clotting, may begin in the blood in the line between the blood vessel and the collection bag which would change the quality of the collected blood. For this reason the blood collection service stipulate a minimum acceptable collection blood flow rate. Typically, 4 to 5 units of blood are collected from individual animals. At the end of blood collection the pig will be killed with an administration of an overdose of anaesthetic without recovery of consciousness.

What are the expected impacts and/or adverse effects for the animals during your project?

These are all short-term studies conducted over one day under non-recovery anaesthesia. Beyond the initial injection of sedative and subsequent induction of anaesthesia the pig will be unaware of any further impacts and adverse events. Impacts (under anaesthesia) can include tissue damage and low blood pressure.
Expected severity categories and the 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 pigs will be non-recovery severity. After sedation by intra-muscular injection and induction of anaesthesia protocols are all conducted under 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?

These studies examine the complex interaction between body systems e.g. cardiovascular, respiratory, inflammatory and clotting. It would be impossible to assess these integrated responses without using whole living animals.

It would be possible to conduct some anatomical studies using cadavers (including human cadavers), but adverse responses relating to tissue/organ function could not be investigated without a living system.
Techniques using living organs or isolated cells can be used to screen drug action and for some studies of local mechanisms. Our research question requires an interaction between several body systems, and the whole body responses to trauma, the only model that can be used are whole living systems.

Which non-animal alternatives did you consider for use in this project?

We also monitor the published literature very carefully before embarking on any trial (human or animal), to ensure that the information we seek is not known already.

Alternatives Human patients

Wherever possible studies to assess treatments of human trauma are best conducted in human patients. These are conducted as clinical trials after an assessment of their safety for the patients, and rigorous ethical examination. We do support and collaborate in trials of this nature when appropriate.

Human volunteers

Wherever possible we have been developing models in human volunteers. These models include ***brief*** periods of simulated significant blood loss (enough to make a volunteer approach the point of fainting) and simulated trauma with the use of tourniquets. It must be stressed that the techniques are rigorously assessed to ensure that they do not cause harm, and the studies are conducted with the volunteers' informed consent. We conduct studies of this nature only after first obtaining a favourable opinion from an independent ethical and safety review from a recognised UK Research Ethics Committee, and where appropriate, consent from the UK national Medicines and Healthcare products Regulatory Agency. The models do not cause lasting harm because the periods of simulated blood loss and trauma are very brief, and are completely reversible. Because the period of simulated blood loss and trauma are so brief they cannot replicate the more profound responses that are also necessary to develop new treatments such as those addressed in this licence application The human volunteer studies are being used to determine the impact of mild levels of injury, assess drug action and methods of better assessing blood loss in a pre-hospital setting.

Why were they not suitable?

Human patients

The clinical trials discussed above are suited for studies to compare a 'standard of care' against a new treatment for which there is strong evidence that it might be better. This supporting (pre-trial) evidence is often based on animal studies such as the ones presented in this application, and hence can be a pre-requisite for a human trial. Studies

such as those presented in this proposal are designed to find the limitations of treatment (i.e. when one treatment fails or is inferior to the 'standard of care'). To knowingly place a human patient in a position where the treatment might fail, to determine its limitations (point of failure), is unethical. Furthermore, to obtain the granularity of information that we need to evaluate reasons for failure, and how to develop new protocols, we need very detailed sampling protocols. This would interfere with patient treatment when the patients are at their most fragile i.e. the study would cause harm to some patients. Therefore, trials such as those presented in this proposal cannot be conducted on human patients.

Human volunteers

It would be unethical to model severe injury in human volunteers as this is very likely to cause lasting harm.

In summary, the new treatments that are the subject of this licence application need to be evaluated in anaesthetised animal models before a clinical trial on human patients.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimate of up to 350 pigs is comprised of up to 200, used in 3-4 studies over the 5 years, each study requiring approximately 50 pigs. The remaining up to 150 pigs are for donation of blood to produce blood products for these 3-4 studies.

The severity of the model of trauma we use to answer any particular question is adjusted in the model development and pilot phases of the study. The model development and pilot phase could require approximately 10 pigs to refine the model so that it would be possible to detect statistical and clinically significant differences with group sizes of approximately 10 animals. This is based on a detailed understanding of the clinical problem, the physiological response and statistical advice.

A planned internal pilot analysis is conducted once group sizes of 6 have been attained in each group in order inform the main study sample size. Over the past 20 years, our research team has provided clinically relevant, statistically robust, answers with group sizes of approximately 10 animals per group.

We have certainty regarding the treatment strategies that need to be compared in the first of the studies, and a clear idea of at least some of the likely treatments in the second study. In the first study we will compare blood products such as mixtures of plasma and red blood cells (which are emerging as standards of care), plasma without the red cells (which would be easier to transport and is being advocated by some groups) and products such as cold-stored whole blood which is not currently a standard of care in pre-hospital medicine. There are circumstances where cold-stored whole blood may be an ideal fluid for resuscitation, but there are other circumstances (injury types) where it may have a significant deleterious effect. The purpose of the first study is to assess the potential

benefits and drawbacks of all of these treatments to suggest the best one for particular types of injury. The second study will look at alternative solutions to providing oxygen to tissues in pre-hospital patients. It is anticipated that this will take approximately 2.5 years to complete. The questions to be addressed in the third (and possibly fourth) studies will be dependent on answers from the first two studies, the prevailing clinical opportunities at the time (new and potential treatments and concepts are being developed all of the time), and the clinical concerns as they develop in line with world events and the role the UK plays in these. New studies will be conducted in the spirit and within the severity limits of the extant licence, and any significant changes will only be conducted after the granting of any necessary amendments to the licence. All new studies will be reviewed in terms of clinical imperative, scientific robustness and ethics before they are initiated.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

If several treatments are being assessed that require the same control group, experiments will be designed wherever possible (within the constraints of reporting timelines) to allow the sharing of control groups between studies, and hence reducing the overall number of animals required.

An internal pilot will be carried out in order to assess the variability of the outcome of interest. This will be used to inform the power calculation to determine the sample size for the remaining animals required in the main study in order to determine if there is a statistical difference between fluid types at an appropriate alpha level of hypothesis testing. This approach has been discussed and agreed with our statisticians.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Pilot studies are used to refine protocols and models of trauma. These protocols and models are fine- tuned to produce a clear (statistically robust) answer with the fewest animals possible (usually group sizes of approximately 10 animals).

The studies are conducted by a multi-disciplinary team so that several relevant body systems are studied simultaneously, reducing the need to conduct similar studies to address additional body systems.

Tissues are collected post mortem and are shared to address additional questions, thereby avoiding the need to use additional animals to answer those questions. For example, we are currently researching the effects of systemic trauma on nerve function. By harvesting post-mortem nerve tissue from our resuscitation studies (which focus on haemodynamic and immunological responses to trauma and resuscitation) we are able to conduct a study on nerve function with minimal additional use of animals.

We also extensively harvest post-mortem tissue which is kept for future projects (as yet undefined) so that as far as possible we can conduct those with minimal use of animals.

Finally studies such as these ones generate a large amount of physiological data. These data are used not only to answer the questions being addressed by the study itself but also kept in a repository where it can be used (or combined with data from other studies) to address additional questions with minimal if any additional use of animals. Some of these additional analyses are conducted in-house, while others are conducted by external

groups who approach us for data.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

All the studies in this licence are conducted under non-recovery anaesthesia, to minimise distress and eliminate suffering.

Why can't you use animals that are less sentient?

Pigs are the least sentient species consistent with the aims of the study and the requirements of the protocols. That choice is driven by the degree of monitoring and granularity of sampling needed as well as how close the model is to the human condition being investigated. Suffering is minimised by the use of non-recovery anaesthesia.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All studies are conducted under non-recovery anaesthesia, which is the most refined way to conduct these studies.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The studies are conducted under non-recovery anaesthesia, which is the most refined way possible of conducting studies of this nature.

Future studies may need studies to be conducted over longer timescales to assess the development of, for example, organ damage and response to therapy. We already use techniques such as transcriptomics and pathology to predict responses beyond the timescale of the experimental study. As a research team we are actively investigating ex vivo systems (in house and by collaboration) so that the in vivo element can still be conducted under non-recovery anaesthesia.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

As Project Licence Holder, and an active researcher, I routinely maintain a watch on the NC3Rs website. At our organisation we have an active programme of education with regular emails sent to all licence holders by a senior Animal Technologist who maintains a watch for developments and conferences that relate to the 3Rs, covering for example items from the NC3Rs, LASA, and IAT (abbreviations defined below). This is a very effective way of keeping abreast of developments relevant to individual programmes of

work. I have active engagement with the NACWO covering my area of research, and the NVS who maintains regular contact with the senior team responsible with the projects.

Abbreviations: NC3Rs, National Centre for the Replacement Refinement & Reduction of Animals in Research; LASA, Laboratory Animal Science Association; IAT, Institute of Animal Technology; NACWO, Named Animal Care & Welfare Officer; NVS, Named Veterinary Surgeon.

42. Kinases in Brain Development and Function

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - 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

Neurodevelopment, Epilepsy, Kinase, Learning, Synapses

Animal types	Life stages
Mice	embryo, neonate, juvenile, pregnant, adult
Rats	pregnant, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to understand the molecular mechanisms that are important for brain development and function. Our goal is to gain an understanding that will lead to treatments for neurodevelopmental and 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?

Neurodevelopmental disorders such as developmental and epileptic encephalopathies (brain malformations) cause severe seizures that are resistant to known treatments. Patients also suffer from general neurodevelopmental delays, often they cannot walk independently or communicate. Several neurodevelopmental disorders are caused by genetic mutations. Development of new treatments for patients suffering from neurodevelopmental disorders depends on our breakthroughs in determining new biological insight that are altered by these gene mutations.

We are particularly committed to studying the roles of signalling proteins called kinases in brain development and diseases. Protein kinases are enzymes that transfer a phosphate molecule to an amino acid on their target protein. This modification alters the function of the target protein. Genetic changes in kinases are found in patients with neurodevelopmental as well as neurodegenerative diseases. We will study the brain regions where we know diseases cause alterations and aim to determine how genetic defects affect brain cells, called neurons. We will use methodologies to determine how neuronal connections and circuit functioning is affected by genes and mutations and we will also aim to develop therapeutic strategies. For example, we will measure mouse behaviour, including their learning, social responses and motor activity, we will perform brain recordings in living animals to analyse their brain functions. We will also use post-mortem tissues to record electrical activity and assess the molecular contents of the brains with various methods including labelling of specific proteins in cells by methods of stainings or analysing molecular contents by transcriptomics or proteomics methods which look into detail the mRNA and protein contents in cells.

One of the kinases we are studying is called cyclin-dependent kinase-like 5 (CDKL5). CDKL5 mutations cause a severe neurodevelopmental disorder with neonatal-onset epilepsy, resistant to medication. These patients have problems with all sensory and motor functions, learning and vision. Our research is continuing to reveal functions of CDKL5 in the brain.

What outputs do you think you will see at the end of this project?

This project will generate novel insights into biological processes that are required for brain development and functioning. It will lead to high quality peer-reviewed publications that will inform the scientific field. We will communicate our findings at conferences. We are studying a rare disease called CDKL5 deficiency disorder. All new knowledge we gather can be used to develop novel therapies for this disorder and more broadly for epileptic disorders.

Who or what will benefit from these outputs, and how?

This work is expected to provide novel information about kinases and other genes involved in brain development. We will publish our results in peer reviewed journals thus advancing scientific knowledge. Biomarkers are molecules that give us information about the type or stage of a disorder. Effective biomarkers, such as blood markers or imaging tests that report disease progression are very important for the development of therapies. Biomarkers can also be used to stratify patients with unknown genetic/environmental causes of disorders into subgroups for subsequent clinical trials. Thus, biomarkers can provide great use for increasing future success rates of clinical trials and provide society with improved treatment options. Our work will generate molecular biomarkers for diseases that are linked to kinases or other genes we are studying. In addition, increasing our understanding of the basic principles of molecular neurobiology is key to future progress in

this scientific area. This work may reveal novel information that can help the development of new therapies as new drug targets can be identified.

How will you look to maximise the outputs of this work?

I have several collaborations in UK and abroad. I am a member of speciality forums, and regularly attend conferences on neuroscience. My lab members present our findings at yearly neuroscience conferences and other broader conferences. All of these interactions shape our future plans and enable us to disseminate knowledge efficiently. In meetings as well as in publications we include "negative data" unsuccessful approaches. All our work will be published in peer-reviewed journals, and we make all possible efforts to make these open-access.

Species and numbers of animals expected to be used

- Mice: 12000
- 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.

Our project's goal is to discover mechanisms that play a role in the formation of connections between brain cells. Brain circuitries that we are planning to work on to understand the molecular basis of their formation cannot be produced elsewhere, culture or organoid models cannot replace these complex, interconnected systems. In other words, brain circuitry relies on the formation of specific connections between different groups of neurons. Such specificity cannot be reproduced in nerve cell cultures or in organoids. Non-animal models cannot replace testing hypothesis in animals, even though these hypotheses may be generated by the non-animal work. Nevertheless, much of the work in this project is to be done using tissue samples. Prior work from other groups and our work in nerve cell cultures supports this project. Mice and rats are species that are highly suitable for studying the brain development and circuitry of a mammal. Brain circuitry of rodents sufficiently resemble that of humans allowing us to model neurodevelopmental disorders in rodents. In addition to tissue samples, we will be able to employ the well-established behavioural and brain recording paradigms to assess the roles of genes that we are studying. Rat neurons in cultures are more robust than mouse primary neuronal cultures, meaning that we obtain highly consistent, healthy neuronal cultures from rats than we do from mice. This is why we regularly use primary rat cultures for experiments that do not require genetically altered mice.

Typically, what will be done to an animal used in your project?

The majority of the mice that are generated in this project will be collected before 1 month of age and their brain tissue will be used to examine brain structure, protein content, and synaptic development. Some of the mice will be used to collect tissue from, we will then record electrical activity in tissues to determine any changes in brain function.

Some animals will undergo surgery to implant an EEG device to record electrical activities

in awake animals. Most mice will be generated in breeding protocols 1 or 2, these mice may be used for post- mortem tissue collection, or majority of the mice will experience one of the protocols (3,4,5,6,7 or 9). Occasionally, mice that have been in utero electroporated as pups (protocol 3) or had brain injections as neonates (protocol 4) will experience one of the protocols (5, 6, 7, or 9). Therefore, some mice which had brain injections will undergo surgical implantation of EEG devices.

Rarely, at newborn ages (0 to 2 days of age), animals will undergo brain injections of virus or pieces of genetic material interfering with specific gene expression into their brain. These experiments are necessary to determine which genes play roles in neurodevelopmental disorders as it allows us to access neurons during development of the circuitry.

Some animals may receive injections of a small volume of substance under the skin of the neck, in the belly or in the muscle of the hind leg in the process of inducing anaesthesia, or pain relief medicines or for other purposes such as injection of labelling substances.

Some animals will undergo standard behavioural experiments which may cause only slight discomfort. Rarely, different cohorts of animals will be tested for their learning and memory using slight electrical shock to their foot. The fear conditioning protocol is a widely used assessment for hippocampal learning and memory retention. It is a rapid learning protocol and requires induction by fear. Therefore, rewards cannot be used to study learning by fear-conditioning paradigm.

Rarely animals will be given injections to induce epilepsy, which incapacitates the animal and therefore animals are observed continuously during seizure induction. A proportion of these animals will have EEG devices implanted on their head to record electrical activity in the brain. These experiments will determine genes involved in the epilepsy disorders we are investigating and will determine potential treatments for these disorders.

What are the expected impacts and/or adverse effects for the animals during your project?

During behavioural experiments animals may experience stress which we plan to minimise through gradual habituation to new environments. Animals may experience pain during injection of substances which typically lasts for minutes. Animals will experience pain and distress after surgery, which we aim to minimise with pain killers and careful monitoring, and which typically resolves after a few days.
Induction of epilepsy can rarely cause mortality. In vivo recording or imaging experiments require surgery for mounting head-bars. Electrodes inserted in the brain may become detached or move away from their original position. Cranial window may become detached.

Expected severity categories and the 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 - 70%
Moderate - 30%
Severe - 0%

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?

Our project's goal is to discover mechanisms that play a role in the formation of connections between brain cells. Brain circuitries that we are planning to work on to understand the molecular basis of their formation cannot be produced elsewhere. Valuable data can be obtained from cells and non-animal studies, including organoids, and these are used to carefully design experiments. However, non-animal models cannot replace testing hypotheses, that may be generated by the non-animal work, in animals.

This is because in organoids and cells specific connections of the brain circuits that we want to study are not reproduced. Much of the work in this project is to be done using tissue collected from animals. Prior work from other groups and our work in mammalian cells supports this project.

Before embarking on any animal experiments, we will collect as much evidence as possible to determine whether a candidate genetic or environmental manipulation has a reasonable chance of success and providing information within living organisms. Evidence will be collected from our own experiences and previous results, as well as by surveying all the publications from cell studies and animal models. Our work specifically required analysis of mouse behaviour and seizure susceptibility aspects that cannot be reproduced without use of animals.

Which non-animal alternatives did you consider for use in this project?

We considered cell cultures, including Human induced pluripotent stem cells (iPSCs), mathematical and computer simulations, and behavioural studies in human volunteers. We have also considered brain organoids and organ on chip systems. I used PubMed to search for keywords of the kinases that we are studying and analysed previously published information in cells.

Why were they not suitable?

Our objectives cannot be achieved by non-animal alternatives alone. In my lab we can use mammalian cell cultures and human neuron cultures for studying aspects of brain cell development. We get valuable information from cell cultures and these help us design our experiments. However, cell cultures have major limitations, they cannot recapitulate an accurate brain circuit connectivity and neurons in cultures do not reach a fully developed state as they would be in the organism. Cultures also cannot be used to assess behavioural alterations of a living organism.

Mathematical and computer simulations are not helpful for our investigations as we are aiming to uncover novel genes and molecular components. Existing gene expression databases will be consulted where needed.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 used our previous research to estimate the number of animals. For most experiments concerning collection of tissue, 3-5 animals per experimental group will be sufficient to obtain meaningful data.

This is because we expect very low variability between individual mice with the same genotype Our previous molecular studies have revealed that our effect sizes are in general much larger than any variability between individual mice. We use crossing strategies to obtain littermate controls with experimental animals.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

I have more than 12 years of experience in working with transgenic mouse colonies. Where possible, I will use online tools such as the NC3R Experimental Design Assistant to design the experiments with the minimum number of animals needed. We employ basic design principles such as, https://eda.nc3rs.org.uk/experimental-design We use homozygous breeding where possible to limit the surplus animals because of complex genetic crosses involving two or more genes. However, littermate controls are essential for our experiments due to variation between colonies. We blind the experimenter to the treatment/ genotype of the animals, we use both genders where possible. We ensure that we control for gender, litter and age during our experiments.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Where possible we share tissues from our animals with other researchers in our institute, we have done this for rats as well as mice. When we generate primary neuronal cultures, we communicate the date of the culture with researchers in different labs and share the cultures (tissues) as needed. We have also cryopreserved our mouse lines and we only breed mice when necessary. We store collected tissues in -80°C freezers in different sample sets. This practice allows us to do multiple experiments from the same cohorts of animals by preparing materials from different sample sets for different experiments. We have optimised various methods for characterisation of protein or genetic material content to determine the minimum amount of brain tissue needed for successful experiments. We use this information to divide tissue into multiple sample sets for future use. This practice minimises the number of mice that we need to breed and collect. Pregnant rats will be ordered when needed, there will not be a surplus from breeding rats. Rat embryos will be used to generate cultures from brain cells in dishes, these will be used to study roles of

different genes in neuronal development and function.

Rat cultures are more robust, healthy and less variable, thus we use rat embryos for some our work that do not require transgenic mice.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use mice and rats in our experiments. The aim of this project is to determine molecular pathways that regulate brain development. In order to study the role of individual genes and signalling pathways, we will generate genetically altered mice. We will collect tissue for our analysis and perform behavioural tests.

We will also use established mouse models of neurodevelopmental disorders. For example, for CDKL5 deficiency disorder we will use a well-established model where CDKL5 gene is deleted in mice. Mice will rarely be subjected to surgical procedures, for example implanting devices. In these cases, anaesthesia will be used, and aseptic techniques will be followed.

Surgery is necessary to allow access to the brain. We will use the least invasive surgery method suited to answer our scientific question. Good surgery techniques and pain killers after the surgery reduce the pain and suffering to the animals
Some mice will be subjected to mild behavioural protocols and rarely learning may be measured by mild electric shock applied to the foot to induce fear- associated learning.

Rarely mice will be subjected to seizure inductions by injection of epileptic drugs. Mice will be monitored constantly during this protocol.

Rats will be used to collect tissue and generate neuronal cultures. Rat cultures are more robust, healthy and less variable; thus, we use rat embryos for some of our work that do not require transgenic mice.

We use state-of-the-art techniques for the biological equations we are investigating. We constantly look out for new developments to use more sophisticated techniques to obtain better data with less invasive methods.

Why can’t you use animals that are less sentient?

Species that are less sentient cannot be used to study brain development of mammals. There are genes and importantly brain structures that are specific to mammals. For example, zebrafish or drosophila (fruit fly) do not contain a specific CDKL5 gene and cannot be used to model cortical development. To examine animal behaviour such as motor functions, learning and memory we cannot use animals under terminal anaesthesia.

We need to evaluate these behaviours to understand how genes involved in brain development impact on mouse learning.

In my lab we can use mammalian cell cultures and human neuron cultures for studying aspects of brain cell development. We obtain valuable information from cell cultures and human induced pluripotent stem cells as we differentiate these in neurons. However, these cultures all have major limitations, they cannot recapitulate an accurate brain circuit connectivity and neurons in cultures do not reach a fully developed state as they would be in the organism. Cultures also cannot be used to assess behavioural alterations of a living organism.

Similarly, simpler non-vertebrate organisms are not suited to reproduce the complex behavioural alterations and they cannot be used to model learning and behaviour phenotypes that are being investigated in mammals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Most mice will be killed in order to collect tissue for analysis and will not be subjected to surgeries or behavioural procedures.

Some mice will undergo surgery, these will be done under anaesthesia and aseptic conditions and appropriate analgesia to alleviate pain and discomfort and reduce the risk of infection. Surgeries will be done in clean facilities to reduce the risk of postoperative infection. These animals will be monitored regularly after surgery, appropriate analgesia will be used.

To reduce the stress associated with behavioural experiments, injections or blood draws, animals will be habituated to being held in the hand of an experimenter. Moreover, injection volumes will be as small as possible. Suitably small needle sizes will be chosen, in accordance with current best practice.

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 to increase animal welfare.

We will ensure that in behavioural assays and fear conditioning animals are habituated in their environment prior to experiment. We use state-of-the-art methods during seizure inductions and animals are continuously monitored during this protocol.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the best practice guidelines provided by NORECOPA and the NC3Rs (e.g. for blood draws or for husbandry), whenever applicable.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will 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 to laboratory animal welfare (@RSPCA_LabAnimal).

43. Developing therapies for neurodegenerative 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

Cell Therapy, Gene Therapy, Parkinson's disease, Huntington's disease, Novel Therapeutics

Animal types	Life stages
Rats	neonate, adult, juvenile, embryo, pregnant, aged
Mice	neonate, adult, juvenile, embryo, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Neurodegenerative diseases such as Alzheimer’s disease (AD), Parkinson’s disease (PD) and Huntington’s disease (HD) and stroke are characterised by profound progressive disability of the patients and increased care burden for the family and society. In most cases these disorders are untreatable and increasingly common within an aging population. As a consequence there is a major international investment in research seeking to develop new drug, cell and gene therapies to alleviate symptoms, or to halt or reverse the progression of disease.

The overall aim of the project is to develop new therapies to treat neurodegenerative conditions. To achieve this, we seek to:

Objective 1: Understand the neurobiology underlying neurodegenerative disease

Objective 2: Develop novel, behavioural tasks to improve the translational value of the research

Objective 3: Develop representative animal models of degenerative disease and explore side effects of treatment

Objective 4: Evaluate potential new therapeutic and regenerative therapies in animal models

Objective 5: Understand the mechanisms underlying potential and new therapeutic strategies to continue to improve 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?

This work is important because it allows us to:

1. Learn more about how and why neurodegenerative diseases arise

2. Use knowledge about how/why diseases occur to develop novel treatments or cures

3. Refine an effective treatment for a neurodegenerative disease, in order to bring it into the clinic for human use

4. Reduced disease burden on individuals and society

What outputs do you think you will see at the end of this project?

Novel data: We will generate novel data related to the treatment of neurodegenerative diseases.

Generation of new therapeutics: We are seeking to develop new cell and gene therapies for neurodegenerative diseases. We have one cell therapy product and one gene therapy product in development, which we aim to bring to clinical trial.

Dissemination of data: We will generate scientific publications from these data.

Who or what will benefit from these outputs, and how?

Long-term (3-10 years), the beneficiary is ultimately the person with the neurodegenerative disease, their families and their carers. Development of novel therapeutics should improve peoples' quality-of- life and health status. Society will also benefit from reduced financial burden due to the cost of treating and caring for people with these devastating diseases.

In the shorter term (0-3 years), we will advance our understanding of how to develop these therapeutics and communicate these protocols/data to fellow scientists via publication and conference presentation. As we develop refined animal models and behavioural tasks, we

will also disseminate these data to ensure other scientists are able to improve their research practices and implement the new tests.

How will you look to maximise the outputs of this work?

Dissemination of research to the scientific community:

The novel data collected over the course of this PPL will be communicated to the scientific community through publication in scientific journals, conference presentations and deposition of data in repositories for other scientists to access.

Dissemination of research to the general public:

We always strive to ensure the general public, and patient groups in particular, are able to learn from the data we collect. To achieve this, we have held open days for patients to visit the lab and we have given talks to patients in the context of local charity groups (e.g. Parkinson's UK local groups and the European Huntington's disease network).

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 recognize 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, Sweden, France, Germany, Italy, Portugal), 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: 12000
- Rats: 8000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The basis of this application is seeking to understand and to treat neurodegenerative diseases. The use of animals is required as valid models of human disease, to assess the functional impact of the disease and to determine the impact of treatments on motor/cognitive behaviour, before treatments can be transferred to a clinical setting with humans.

Rats and mice are used as the least sentient mammals to model the relevant systems and functions disturbed in human neurodegenerative disease. These species provide the most

extensively validated models for addressing the physiological, anatomical and behavioural functions under investigation.

We typically use adult animals in our studies, since we are studying diseases that most often affect the adult or aging brain, such as Parkinson's disease. However, there is also good rationale for working with younger animals in some experiments. For example, new theories suggest that Huntington's disease actually has both a developmental and a degenerative phase, making it important to test our therapeutics at different ages/stages of disease progression. Another example of when younger animals may be used is to compare the efficacy of our treatments in the young and aged brain, since recent data have suggested that cell therapies maybe most efficacious if applied in younger patients.

When we work with genetically modified models of disease, we strive to include both male and female rodents for studies that assess disease progression (e.g. assessing behaviour or biomarkers of disease) or that test therapeutics (to ensure equal efficacy in both sexes). We tend to use female rats for long term (1+ year) studies of human cell therapies for pragmatic reasons. Firstly, animal welfare is improved with the use of female rats because they do not fight and do not need to be separated for single housing, so we can maintain communal housing long-term. Secondly, their weight is stable across their ages, so behavioural testing at younger ages (e.g. 2-4 months) yields comparable data to behavioural testing at 12-18 months of age. Male rats are heavier and their behavioural profiles change (or some tests become unusable due to their size), making longitudinal testing challenging.

Typically, what will be done to an animal used in your project?

In order to study diseases in rodents, it is often necessary to use an initial intervention to create/cause the neurodegenerative disease to manifest in the rodent. The two main ways to create these models of disease are to: (1) use a surgical procedure or an injection, in order to infuse a substance that will degenerate particular cells in the brain; or (2) to breed animals to harbour a particular genetic mutation that causes the neurodegenerative disease or related cellular dysfunction. These ‘diseased’ animals will then be studied using a combination of behavioural tasks, non-invasive brain imaging (such as PET), brain recording and/or physiological tests (such as blood sampling).

Some animals will then receive ‘treatment’ interventions, to determine if the disease can be cured or improved using the new treatment. This stage can involve: (1) another surgical procedure (to inject/infuse a drug, cell or a gene therapy); and/or (2) injections, or other forms of administration, of drug substances. However, in some cases, the treatment interventions are mild and only include undertaking exercise or cognitive training. Some treatments, such as those using human cell therapies, will require the animal to receive daily injections of an immunosuppressant drug; this is perhaps the most intensive treatment situation that an animal could be placed in. After treatment, the animal will continue to be studied to see if any improvement occurs, using behavioural tasks, non-invasive brain imaging (such as PET) and/or physiological tests.

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.

In some cases, experiments are designed to optimise the rodent model itself or to optimise a behavioural task, with the subsequent aim of studying the disease process or a treatment using the new model or task.

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 or the presence of a genetic mutation.

For needle injections, transient pain at the sight of injection is the primary side effect.

Genetically modified mice that harbour mild mutations (such a fluorescent tags or Cre) should not experience any adverse effects. Animals that harbour mutations associated with disease/cellular dysfunction typically have no impairments evident at younger ages, but if kept into older age will often start to manifest elements of the disease. This may include features such as weight loss and motor slowing, cognitive deficits.

Surgical interventions are typically neurosurgeries to infuse a toxin or viral vector (to induce disease) or neurosurgeries to infuse therapeutic interventions (drug, cell therapy, gene therapy). Typically, animals recover well from these and no overt impact is observed. In some cases, however, animals may experience transient weight loss in the days post-surgery, lethargy, or motor abnormalities (since we are studying a part of the brain critical for motor function). 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’. Behavioural testing is not aversive for the animals and is appetitively motivated. Needle injection, non-invasive imaging and mild genetic modifications are typically classified as mild. Surgical interventions and genetic modifications that result in progressive disease pathology are, at most, 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.

We work with both mice and rats and would expect approximately 50% of each cohort of animals to be diseased (either through infusion of a toxic substance or by virtue of harbouring a genetic mutation).

The remaining wild-type or control rats are typically exposed to the same treatment conditions, so some of these will undergo procedures related to the treatment intervention. For example, a typical experimental design might use 50% wild-type and 50% mice that harbour a Huntington's disease mutation. Then, 50% of each genotype will receive an injection of a drug, while the other 50% will receive a control injection of sterile saline.

However, when we are using these same mice to study the development/progression of the disease, the mice might be bred (50% wild-type, 50% genetically modified) and they might undergo some non- aversive behavioural assessments, prior to being culled and tissues harvested for molecular analysis.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The basis of this application is seeking to understand and to treat neurodegenerative diseases. While we do use alternative strategies wherever possible to understand disease and to develop treatments (such as cell models), the complexity of the brain cannot currently be mimicked in the cell culture dish. Moreover, rodent models are necessary to identify potential side effects, develop dosing strategies and ensure safety before treatments are taken into the clinic and trialled in humans. Therefore, the use of conscious living animals is required as valid models of human disease. Where in vitro or ex vivo assays will suffice, these approaches will be used to replace live animal studies. However, the living, functional brain will ultimately be required to assess the functional impact of the disease and to determine the impact of treatments on motor/cognitive behaviour, before treatments can be transferred to a clinical setting with humans.

Which non-animal alternatives did you consider for use in this project?

We have actively used cell culture to model specific brain cells in a dish. In these experiments, it is possible to answer simple questions or derive preliminary data on the impact of a new drug or viral vector on the cells. However, where these data look promising, it is pertinent to move to animal models to validate these effects and to test the drug/vector in the context of the more complex brain system, and to study the whole animal for evidence of side effects.

We have also used ex vivo imaging assays to study disease-induced changes in the brain, without subjecting animals to experimental procedures (such as anesthesia exposure).

Why were they not suitable?

To some extent, these alternatives are indeed effective and we will always seek to use them when feasible. Since we are trying to understand human disease and develop therapeutics, these models can be limited in the data that we can obtain. Ultimately, we may need to test the efficacy of a therapeutic on motor or cognitive function, or assess potential treated-induced side effects, which is ultimately only possible in a whole animal 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?

These estimates are based on (1) using data from my previous/current PPL to predict future use and
(2) calculations associated with anticipated grant applications from my research group.

Previous/current PPL: In P49E8C976, larger numbers of mice were used due to the breeding, maintenance and experimental use of several GA mouse lines. ~5000 mice were reported in Home

Office returns by year 3 (out of 5). Rats are also a critical species in our experimental field and we regularly purchase wild-type rats to create inducible models. We have recently established a rat GA model of Huntington's disease, which we anticipate using for multiple experiments over the next few years.

Current/Anticipated grant applications: Our research programme ultimately aims (1) to understand neurodegenerative diseases and (2) to develop novel therapeutics, to bring to the clinic. Ultimately, this will require many thousands of animals as we continually refine our cell/gene therapy products and develop 2nd and 3rd generation products. For each novel product, we require 1-2 experiments to establish functional efficacy of the product (i.e. the ability to improve motor and cognitive function), 1-2 experiments establish synaptic connectivity, 1-2 experiments we much establish safety, 1-2 experiments to fully characterise the product after it has been in situ for months, and 1 experiment to identify any side effects of the product. Additionally, we are working in two different diseases, which affect similar brain systems (i.e. the basal ganglia), but often have unique challenges associated with each disease and each requires regeneration of a different cell type. Finally, we are always trying to further out understanding of the diseases in order to develop ever more refined therapeutics.

Therefore, I would estimate ~20k rodents would be required to meet our ultimate goals. However, not all of these studies will be able to be completed within the 5 years of this Project Licence.

Currently, we have a large MRC Programme Grant, which is funded until 2025, which is anticipated to require ~1000 rats (50% wild-type Lister-hooded, 50% transgenic

Huntington's rats). I also have funding from an industrial partner for an experiment that requires ~200 rats over 1 year, and an MRC grant that requires ~100 rats. We also have 3 independently funded PhD students who are working with animals (~50-100 rodents/year/student). It is anticipated that we will apply for a further 3-6 grants per year. Whilst it is challenging to predict how many will be granted, we use our current rate of funding to help us pragmatically determine how far we are able to develop our knoweldge of the diseases and our therapeutics over the next 5 years.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We always seek to reduce the numbers of animals used in any project by relying on in vitro assays, predominately the culturing of cells, to obtain data wherever possible. 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 have used the NC3Rs Experimental Design Assistant, which we found useful in our recent successful application to the N3CRs and we intend to continue to use this.

In many experiments we use a "within-subjects designs" in which the same animal might receive both the treatment condition and the control condition (e.g. on one day the animal might get the drug but then on the next day it will receive the control solution but lacking the drug). We also have "within- subjects design" models, for example, the unilateral 6-OHDA lesion model manifests motor impairments in the contralateral paw, while maintaining normal function in the ipsilateral paw. This reduces the number of animals required and also reduces the amount of variability in the experiment overall which then, in turn, further reduces the number of animals that we need.

In many of our experiments we will collect both behavioural data and other data, such as physiological data, recordings of brain activity or harvesting of tissues. 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 extensive development and testing of the treatments in vitro/in cell models prior to utilising the in vivo/rodent models. This way, we aim to increase the power of the experiment by utilise only the most optimised treatment preparations to test in rodents. By minimising variation in the treatment, we can use the smallest number of rodents that still allows sufficient sensitivity to detect differences that results from the experimental intervention.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the

mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 models of disease or related cellular dysfunction. These can be induced through expose to a neuroactive substance (e.g. toxin, viral vector or similar substance), or through use a genetic mutations. Some mutant models may harbour non-harmful mutations, such as Cre- or fluorescent tags, or breeding crosses may occur with rodents with mutations that may alter disease onset/progression, such as DNA repair genes. We also use immunocompromised rodents if they receive cell transplants and these can be chemically immunosuppressed, or genetically immunocompromised.

We consider carefully the model to use, depending on the experimental question at hand, but we are also cognizant of the impact on the animals. In order to ensure best practices, enhance animal welfare
- and also to improve the quality of the experiment - we may seek to create models with some evidence of disease, but ensure that the animals are well enough to (for example) perform appetitively motivated behavioural tests or undergo imaging.

Why can’t you use animals that are less sentient?

We can't use less sentient animals such as flies, worms or fish because their brains are not similar enough to human brains, and they don't exhibit the same behaviours as humans. As our goal is to model the disease process and to create novel therapeutics, it is important to work in models that are sufficiently translational as to be an effective means of progressing the research.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Operations on the brain are performed carefully and in clean, well-stocked surgical theatres, under strict aseptic conditions. The animals are given pain killers during the operations until they have fully recovered. They are also given highly palatable food to help them recover. The animals are monitored very carefully for at least 7 days after any surgery. Generally, soon after the operations you would not be able to tell the difference between the experimental animals and controls in terms of the way they behave in their home cages. Similarly, the genetically modified mice can be indistinguishable from normal mice when observed in their home cages. It may be only with the sophisticated tests of cognitive function that you can begin to tell them apart. As animals age, more disease phenotypes become evident, or as animals are treated longer term with drugs such as immunosuppressants, side effects may eventually emerge. These animals are monitored very closely by the personal licence PIL holder, the project licence PPL holder, the NACWOs Named Animal Care and Welfare Officers and the NVS. Animals are removed from the study in advance of significant clinical signs. During behavioural testing, animals are monitored very carefully. Indeed, many of our behavioural experiments involve us watching the animals perform. The animals will get extensive handling and training to perform the tests.

We also continuously consider how to refine our models that experience immunosuppression. We have used daily injections of cyclosporine for decades and have used improved formulations of the drug that are increasingly less aversive. We have recently established the use of athymic rodents in the lab, which can be used when extensive behavioural testing is not required, in lieu of chemical immunosuppression. We have also recently been awarded funding from the NC3Rs to test 'local' (intracerebral) immunosupression using Tacrolimus-releasing microbeads. These beads will be infused alongside the cell transplants and we hope that this method will removed or reduce the need for any systemic imunosuppression. Thus, we are continuously refining these models, with animal welfare the highest consideration.

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?

I am Deputy Chair of my home establishment's Animal Welfare and Research Panel. On this panel, our local NC3Rs representative gives us regular updates and I then communicate this information to all the users of the animal facility in my department. Thus, I am generally the most well informed member of the department in terms of NC3Rs. Additionally, my team and I keep a look out for latest information on the NC3Rs website and discuss this at our weekly lab meetings.

I also engage with UnderstandingAnimalResearch.org (and have even written articles for them myself to discuss issues related to the use of animals in research). They regularly provide updates on new advances in the 3Rs.

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. For example, I have closely followed the development of a novel alpha-synuclein viral vector model for Parkinson's via Pubmed/published literature. I recently brought the model to my own lab through direct engagement with the research group in Sweden that has now spent years refining the model. We implemented their newest version of the model, which they subsequently published a report to demonstrate was their most reliable model, with the least variability and least impact on animal welfare.

44. Improving pharmacokinetics of antimicrobials through formulation and tissue targeting

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

Antibacterials, antivirals, drug delivery, formulation, pharmacokinetics

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Our overall aim is to increase the effectiveness of drugs by making new formulations that improve their pharmacokinetics (this is what the body does to a drug, it refers to the movement of drug into, through, and out of the body) and tissue distribution of drugs. New formulations that stop or reduce infection and reduce inflammation that are directly targeted to certain parts of the body including the lung, brain and liver.

This should allow current drugs to successfully treat more infections, and allow the potential of new drugs to be fully realised.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The project largely aims to develop better treatments for ‘Emerging Infectious Diseases/Pathogens’ as defined by the National Institute of Allergy and Infectious Diseases (USA) and adopted by a number global bodies.

These diseases have a high death rate, an example is Melioidosis; also called Whitmore’s disease, is an infectious disease that can infect humans or animals, it is an infectious disease that most commonly affects people in South East Asia and Northern Australia. It is a serious emerging pathogen and mortality has been reported from 20-50% depending on geographical location.

Therefore, we believe new and improved delivery and targeting of antimicrobials/viral drugs would be beneficial in treating a variety of infections including biodefense and pandemic capable pathogens of concern, by maximising efficacy and minimising toxicity.

What outputs do you think you will see at the end of this project?

This project will produce new information that will be communicated through peer-reviewed scientific publications, conference presentations and patent applications. We envisage this will be translated into new treatments for life-threatening human infections, including Whitmore disease.

Who or what will benefit from these outputs, and how?

Immediate beneficiaries will be scientists working in similar areas. Many of the systems we plan to use to create the new delivery methods are technologies that are already available and the results will be of general use for drug delivery and targeting drugs to specific tissues. Thus our efforts might yield systems and formulation information that can be applied to a wide variety of drugs and other diseases.

Medium term benefits will be addressing an unmet clinical need, improving Human Health and reducing human suffering (benefiting patients, clinicians and the pharmaceutical industry).

Other medium to long term benefits are better treatment options for Emerging Diseases/Pandemic capable pathogens/Security/biodefense. These pathogens cause Emerging infectious diseases that can be defined as infectious diseases that have newly appeared in a population or have existed but are rapidly increasing in incidence or geographic range. Consequently, progress made in treatments are likely to have a large impact. Being able to effectively respond to these threats also protects the economy that can suffer when insufficient medical interventions are available and the only means to control a epidemic or pandemic are through non-pharmacological interventions such as 'stay at home orders/lockdowns'.

How will you look to maximise the outputs of this work?

Results will be communicated to the scientific community through peer reviewed scientific publications and presentations at research conferences. Rigorously demonstrating that an approach is unsuccessful allows its publication or inclusion in a publication with an eventual successful approach. Through a growing collaborative network now comprising funders and collaborators in USA, Canada, and multiple institutions in UK we have the

ability to apply our findings in many models of infection and maximise the potential benefits.

Species and numbers of animals expected to be used

- Mice: 2000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are using adult mice because they are widely used for this type of research and they share over 95% of genes with humans. Working out how a drug gets to the tissue and then leaves the body is very complex, and it is as yet impossible to replicate this in a non-animal model.

Typically, what will be done to an animal used in your project?

In most studies, mice will receive a single dose (to the stomach, the blood stream, under the skin or into a vein) of a newly formulated drug. It is expected that all drugs will already have been tested for toxicity through administration to animals previously (most likely historically) and may even be licenced for use in animals or humans. Blood samples might be taken. Animals will be humanely culled and tissue taken for further studies

In some studies, so we can track where the drug has gone, mice will be injected with a fluorescent marker substance, they will be anaesthetised, placed in a machine (an imaging system) to determine the location of the administered formulation.

What are the expected impacts and/or adverse effects for the animals during your project?

We expect the potential adverse effects will relate to potential harmful effects (toxicity) of drug formulations, but these are expected to be low as only drugs where acute toxicity is known not to be a problem are used. However, some antibiotics in common use are known to cause mild diarrhoea, that could occur for some or all of the potential 48 hour duration of the experiment. Very unlikely adverse effects could potentially occur where the formulation is designed to alter the delivery of the drug to such an extent that it becomes toxic. The likelihood of side effects is reduced as the experiment will use a single dose of drug and only last up to 48 hours. Animals will be weighed at the beginning of the study, monitored twice daily and animals will be euthanised if weight loss goes below 10% of start weight, although this is unlikely in a 48 hour 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)?

All mice are expected to experience only mild severities as we are testing drugs with known toxicities (albeit in new formulations). All experiments will be of short duration (no longer than 48 hours). All animals will be humanely euthanized.

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 ability of the body to breakdown and eliminate a drug is complex and is affected by multiple parameters including the route of administration, multiple cell types and organs, blood pressure and metabolism which are as yet impossible to replicate in any in vivo system.

To determine the anatomical site that a drug reaches is best achieved in an animal that closely resembles humans and has a similar circulatory system and organs.

Which non-animal alternatives did you consider for use in this project?

We currenty use in vitro systems to screen our formulations for stability and cellular uptake and have considered their use for pharmacokinetic studies too.

There are in vitro models that mimic certain aspects of the 'blood brain barrier' an important barrier to cross for effective delivery to the brain. We do plan to use this model as part of a collaboration.

Why were they not suitable?

In vitro systems are not capable of replicating circulation systems of mammals or their complex sequential metabolism as they move from anatomical compartments and tissues.

We do plan to use an in vitro model for the blood brain barrier. However, it does have the limitations above meaning that it is not a direct replacement. However, through comparison of data in the in vitro system with results obtained in the in vivo model, we hope to use it in the future to down select formulations for in vivo studies.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Animals numbers are based on testing 4 studies per year of the study. Each formulation is typically tested in groups of 5 mice at each time point examined and compared with non-formulated drug to provide scientific rigour and confidence in the results obtained. Up to 8 timepoints can be required to track the metabolism and elimination of the drug over the 48 hour time period. This can therefore equate to 85 mice per study (340/year) and 1700 over 5 years. In addition 300 mice might be used for in vivo imaging studies over the 5 years.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We use the smallest number of animals required to provide statistical power to detect a difference between groups. We use the NC3R's EDA. By using an in vivo imaging system we hope to reduce the number of animals use by reducing the need to euthanise animals for tissue and allow us to use a single group of animals to gain infomation at a number of timepoints.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

When control mice are euthanised we often use tissue for other purposes. for example we can use femurs to extract bone marrow for generation of macrophages and dendritic cell cultures.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

These studies are of short duration and 80% of mice will experience mild severity, (the reminaing 20% might exerience moderate severity with symptoms including tiredness or mild diarrhoea). Mice will be used in all studies and will receive a single dose of a drug via injection (intraperitoneally, intravenously or subcutaneously) or orally via gavage. Blood samples may be taken or at the time of euthanasia 15 mins-48 hours post administration.

Why can’t you use animals that are less sentient?

Less sentient animals such as insects or other invertebrates have different circulatory systems and metabolism.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

A number of procedures are in place to refine procedures and minimise welfare costs including single use of needles, approved handling techniques, environmental enrichment. Animals will be monitored following administration for any adverse effects.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

NC3Rs EDA, Local guidelines (based on ECVAM) maximum injection volume.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Information from the NIO, review of the NC3Rs website. I also subscribe to the NC3Rs newsletter and attend our insitutional 3Rs research conferences in SIPBS.

45. Environmental Effects on Fish Physiology and Behaviour

Project duration

5 years 0 months

Project purpose

- Basic research
- Protection of the natural environment in the interests of the health or welfare of man or animals

Key words

fish, environment, climate change, physiology, behaviour

Animal types	Life stages
Zebra fish (Danio rerio)	embryo, neonate, juvenile, adult, pregnant
Salmon (Salmo salar)	embryo, neonate, juvenile, adult, pregnant
perch (Perca fluviatilis)	juvenile, adult, pregnant, neonate, embryo
Brown Trout (Salmo Trutta)	embryo, neonate, juvenile, adult, pregnant
Rainbow Trout (Oncorhynchus mykiss)	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The project will investigate how changes in physiology, due to shifts in environmental conditions and fishing operations, are linked to behaviours such as foraging activity, predator avoidance, swimming performance, social behaviours and group living, and habitat selection.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 natural environment is currently undergoing unprecedented rates of environmental change, largely due to human-associated activities. This is particularly true for aquatic habitats, which even under ideal conditions show much greater variation in environmental factors such as temperature and oxygen availability compared to land-based habitats, and additional threats stemming from over-fishing and rapid climate change. One of the most pressing concerns in conservation is how environmental change is affecting wild animal populations, including their ability to find food, avoid predators, and reproduce. To understand these effects, it is vital that we increase our understanding of how environmental changes affect the internal physiology of animals, and the consequences of these changes for their behaviour. More information in this area will increase our understanding of whether animal populations and species can cope with change and inform potential conservation efforts.

What outputs do you think you will see at the end of this project?

The project outputs will include increased knowledge of how environmental stressors affect fish ecology, with knowledge being shared through the production of research publications. There will also be numerous public outreach projects carried out during the project, aimed at increasing awareness of the threats being faced by wild fish populations and animals in general. Previous work in this area also been has also been used to inform policy regarding regulations for catch-and-release angling, and it is anticipated that this may be another outcome of the project.

Who or what will benefit from these outputs, and how?

Short-term benefits of this project include crucial information on how interactions between metabolic physiology and behaviour in individual animals affect their ability to cope with changing environmental conditions. This information is vital for understanding the role of food requirements, physical activity, and disease resistance in behaviours associated with foraging, predator avoidance, and reproduction. These results will be of immediate interests to physiological and behavioural ecologists and conservationists.

In the medium and longer terms, the results of this project will be beneficial for informing future fisheries management strategies and fishing techniques to minimise the impacts of recreational and commercial fishing. For example, this project will contribute toward predicting the combined effects of climate change and overfishing, including the effects of these factors on the distribution of fish species. Furthermore, the problem of discards from commercial fishing operations and the ability of fish to physiologically recover and resume normal behaviour after discard is a major research priority across the globe. Many regions are implementing bans on fisheries discards but allowing special exemptions for species which show an ability to recover from the stress of capture and release. There are currently almost no data on which to base such decisions but the current project will provide this information through research with the species of interest as well as established model species, from which results are broadly applicable. Finally, it increasingly recognised that fishing can selectively remove specific types of individuals within populations, for example, by removing the largest or slowest moving fish. In turn, this selective fishing alters the individuals that are left to reproduce within the population, affecting which characteristics can be passed on to the next generation. More information on which individuals are most vulnerable to fishing gears will transform our perceptions of

how this process can affect the evolution of fish species, a problem which is currently one of the great threats facing economically and ecologically vital fish stocks. Managers will be able to use data from this project to alter the design of fishing gears design to minimise selection within species and reduce the evolutionary effects of fishing.

Another long-term benefit will be a greater understanding of the factors which allow some species to become invasive when they are introduced into native habitats, and possibly develop methods for controlling the range expansion of alien species. There are also potential applications here for the aquaculture industry where growth maximises profit but trades-off among food requirements, growth, and immune function that can reduce growth efficiency. Another direction for applications is sports medicine; if intense exercise affects physiological traits or the motivation to perform certain behaviours, understanding the mechanisms underlying this would be valuable. Fish are ideal for studying this area because they can be subjected to treatments that are logistically not possible in humans.

How will you look to maximise the outputs of this work?

The applicant has a wide-network of collaborators throughout the international community and this can be used to expand awareness of this work, including contacts with policy-makers. The research group is also extremely active in presenting work at conferences and on social media, which further serve to maximise dissemination of findings. Notably, the research group as also been active in the development of best-practice guidelines for various research techniques and methods for improving research transparency.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 3000
- Brown Trout (Salmo Trutta): 100
- Rainbow Trout (Oncorhynchus mykiss): 100
- Salmon (Salmo salar): 100
- Other fish: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Fish species have been chosen because they perform all of the behaviours of interest and are a key component of natural ecosystems. In addition, the physiological and behavioural traits that fish exhibit are generalisable and can be used for generating conclusions and research ideas for other wild animal species. For most aspects of the project, juveniles will be used due to their ease of holding and because they lack mating behaviours that can interfere with other behavioural observations. For other aspects, however, adults may be used, especially when examining effects of environmental factors on growth or reproduction.

Typically, what will be done to an animal used in your project?

Typically, animals will be exposed to varying environmental factors (e.g. temperature, oxygen availability, light, feeding levels) within their current natural ranges or those

predicted by various environmental change scenarios. Animals will then be observed for behaviour (e.g. social behaviour, foraging behaviour) and various aspects of the physiology (e.g. metabolic rate, swimming performance, growth). Some animals will be tagged for identification using minimally invasive methods (e.g. elastomer, PIT tags). Some others will be implanted with a transmitting tag to track their location and behaviour in the wild using a minor surgical procedure. The duration of the experiments will vary depending on the exact variables being measured but will generally be on the order of days for behaviour and metabolism, days or weeks for growth rates, and weeks or months for the tracking of movements in the wild. Animals may be measured under more than one procedure such that repeatability of measures can be estimated or correlations between traits (e.g. aspects of behaviour vs aspects of physiology) can be determined.

What are the expected impacts and/or adverse effects for the animals during your project?

The expected adverse effects include short-term pain, possible weight loss, and minor disruptions to behaviour including: increases or decreases in activity (depending on the treatment), altered social interactions (including aggression), and altered use of shelter or feeding motivation. These effects will all be short-term in duration (usually minutes or hours). The extent and duration of any adverse effects will be monitored by the researchers and animal technicians, with on going communication and documentation of any unexpected effects. For animals to be released into the wild with acoustic transmitters, the short-term pain or discomfort from the small incision is likely to last hours to days. Fish generally feed normally within hours after tag implantation, but will be observed for adverse effects (altered behaviour, infection) for at least two days before being released.

Expected severity categories and the 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 experienced by the great majority of fish used in this project will be mild since they will only be subjected to benign procedures such as manipulation of environmental conditions (e.g. diet, temperature), observations of behaviour, or measurement of metabolic rate or swimming performance. In some instances, fish may be subjected to simulations of procedures that commonly occur during fishing practices including trawling, air exposure, and handling. The extent of these simulations will not be as extreme as that which occurs in an actual fishing scenario but will be sufficient to elicit variation in the physiological and behavioural responses of interest. Nonetheless, these procedures may be of moderate severity. At the end of procedures fish will be humanely killed. An exception to this are fish that will be returned to the wild after being fitted with an acoustic transmitting tag.

What will happen to animals at the end of this project?

- Killed
- Kept alive
- Rehomed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

All experiments are based on studying the behaviour, growth or physiology of fish, and so there is no alternative to the use of live sentient animals. There are no systems using isolated cells, tissues, or organs that can answer the scientific questions posed in this project. Moreover, many of the experiments will be explicitly studying the consequences of physiological and behavioural variation between animals, and so must use sufficient numbers of individuals to gain a true estimate of the variation present for a given trait. In some cases the characterisitic of interest (e.g. age of sexual maturation) will not be apparent at the start of the experiment, and so the initial sample size needs to be large enough to allow for the uncertainty in the final sample size in each category (e.g. each age at maturation for each sex – most species of fish cannot be sexed when immature). However, the degree of pain or suffering imposed on the animals will be small, since the protocols are generally noninvasive and involve studying the animal in its near natural state.

Which non-animal alternatives did you consider for use in this project?

The ultimate aims here require data on the responses of live animals to environmental factors such as tempertaure, oxygen, or food availabilty. Computer-based models of animal behaviour can, in some cases, be used to generate predictions of how animals might behave or physiologically respond to changing conditions, but these simulations depend on data from live animals to be useful. The applicant and collaborators are currently exploring the use of 3D-printed model animals or video displays to use as a stimulus for some behavioural measures, as an alternative to the use of live animal stimuli (e.g. in the study of various aspects of social behaviour).

Why were they not suitable?

Mathematical models of the effects of environmental factors on individuals, species, and ecosystems not only depend on pre-existing data and assumptions, derived from experiments with live animals, but the findings must then be validated using live animals. This is because the models often incorporate untested assumptions of the behavioural and physiological traits of live animals, or use parameters values for which there is little or no existing data.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Throughout the design of individual studies, literature searches are performed to estimate the expected effect size and variance in the traits of interest such that the appropriate

sample size can be determined. The levels within each experimental treatment (e.g. number of test temperatures or feeding treatments) are investigated prior to the experiment to determine the resolution needed to derive strong conclusions while minimising the number of treatments and therefore animals. The research group also has extensive experience in designing similar studies, and analysing the resulting data, and can use this prior experience and work to estimate predicted effect sizes.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Throughout the design of individual studies, literature searches are performed to estimate the expected effect size and variance in the traits of interest such that the appropriate sample size can be determined. Many experiments will study the variation between animals, and so must use larger sample sizes than are necessary to simply generate population mean values. In many cases, the same data from a particular set of measurements can also be used to examine multiple questions of interest. However, in some cases, fish that have only experienced a mild level of severity when undergoing procedures detailed below, may be re-used in subsequent procedures after being certified as fit by the NVS. Individual fish will only be re-used in this manner a maximum of twice (original use plus one instance of reuse). The reason for this practice is that many of the fish used under this licence are wildcaught and not easy to source, plus they require time to settle into the captive environment. In this way we will minimise the overall number of animals used and will not impose undue stress on wild-caught or experimental animals since virtually all of the work will be non-invasive (mostly observational in nature). By collecting detailed information on individual fish that have been subjected to environmental manipulations we will be able, for instance, to test how exposure to various environmental variables (e.g. temperature, hypoxia) affect physiology and behaviour. Moreover, links between each of these traits can be studied (e.g. metabolic rate and social status). Typical sample sizes per experiment will be up to 200 individuals or 20-30 replicate groups per treatment.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

All experiments in the applicant's research group involve an initial stage of pilot study to not only refine the study techniques but also reduce the risk of study failure and the unnecessary use of animals. In addition, individual studies are frequently based on or compliment predictions derived from mathematical models or computer-based simulations.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Although fish evolved early in the evolutionary history of vertebrates, they display a diverse array of complex behaviours that make them ideal for the proposed work, including various forms of social behaviour and foraging and predator avoidance strategies. Fish are relatively easy to rear in the laboratory, and we have great experience in designing experimental and observation tanks for these species. All species will be held in communal stock tanks when not used in experiments (providing natural social interactions), and due attention will be placed on providing shelters and natural lighting levels.

The degree of pain or suffering will be relatively small, since procedures are almost all non-invasive and involve studying animals in near-natural states. Laboratory simulation of fishing procedures will allow for controlled conditions and immediate response measures that will inform results obtained in the field with larger fish. These experiments will use zebrafish or minnows, which have previously been used for fisheries selection experiments. These species are easily maintained in the lab and their short generation time of zebrafish makes it feasible to produce multiple generations within a reduced timeframe. The fact that molecular tools have been optimised for use with zebrafish permits genetic analysis that would not be possible with other species.

Simulated fisheries handling procedures will in some cases be performed using simulations with species that closely resemble larger commercial species but that are smaller and easier to work with (e.g. zebrafish and minnows). Zebrafish and minnows are a highly appropriate species for studying vulnerability to capture by commercial fishing procedures. They are both moving throughout the water column and interact with the substrate in their native habitats. In this regard they are similar to larger species targeted by commercial fisheries (e.g. cod, haddock). Like these larger species, zebrafish and minnows also are also social, shoaling species, and behave almost identically in response to a simulated trawl net as in underwater video of Atlantic cod with full-scale trawls. Each species resists net entry by attempting to maintain position at the trawl mouth. Zebrafish and minnows maintain a similar vertical position above ground as do cod and haddock ahead of a trawl and are therefore likely to use similar escape routes when evading capture (e.g. below the trawl). For scaled-down simulations of fishing activity using these established model species, fish will be placed in nets for a maximum of 15 min and air-exposed for a maximum of 1 min. This duration elicits a cortisol response in adult zebrafish and minnows, which is similar to that observed in commercially fished species, indicating a representative level of stress but also allows individuals to recover. All fish are expected to recover from these stressors over the course of several hours.

Over the last several years there has been advancements in several methodologies to be used in the current project. For example, our lab has frequently included forms of enrichment (e.g. plastic plants) around respirometry chambers when measuring metabolic rates. We also use automated software for behavioural analyses, which cna provide extremely accurate measures of indices such as movement speed and space use, without the potential stress effects and biases that can come along with livescoring of animal behaviour. Our lab also frequently uses shuttle-boxes for the study of fish environmental sensitivities, which allow individual animals to effectively control their environment, allowing researchers to estimate animal avoidance limits to factors such as oxygen availability or temperature before physiological disturbance is generated.

Where appropriate, analgesics will be administered following tagging procedures, as our lab has established in our published best-practice guidelines on this topic.

Why can’t you use animals that are less sentient?

Specific studies aim to examine the behavioural and physiological responses of post-larval or mature fish to various environmental factors. Therefore, the use of other species or less sentient life-stages would not be appropriate.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The applicant has 23 years of experience in the protocols described in the licence and will provide training and oversight of the use of animals. Over the course of this time, several refinements have been implemented by the research group including holding fish in an enriched environment (e.g. with shelter and substrate as appropriate) and carefully controlling in group sizes to minimise aggression (variable depending on the species). There is also much more attention paid to light levels, with light fading in and out at dawn and dusk to eliminate any startle responses. Holding temperatures and water quality will be carefully controlled and monitored daily. Air-exposure and handling of fish will be minimised at all points, from the collection of fish in the wild to their use in the experiment. Where appropriate, analgesics will be administered following tagging procedures. This represents a significant refinement that has become increasingly used in recent years. The applicant also plans to perform more detailed evaluations of how these analgesics may expedite the recovery of normal behaviour and physiological parameters following tagging procedures. The applicant also plans to quantify how enrichment and the social environment affect the behaviour and physiology of fish in a research environment, to maximise welfare and perform the highest quality research. The named animal care and welfare officer (NACWO) and named veterinary surgeon (NVM) will be consulted regularly and kept informed of our work. In particular, the NACWO will be in daily contact with all fish held in the aquarium facilities, performing daily water quality and health checks.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

General experimental design and reporting guidance will be sourced from the ARRIVE guidelines:
Percie du Sert et al - The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research
(2020). PLOS Biology https://doi.org/10.1371/journal.pbio.3000410

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The Project Licence holder and members of the research team will ensure continued professional development in the 3Rs area through regular attendance at meetings/workshops such the Animals in Science Regulation Unit (ASRU) annual meeting, 3Rs workshops/symposiums held at local or national Research Institutes, and also attendance at local training workshops organised by the NTCO. We also visit the NC3Rs webpage (https://www.nc3rs.org.uk/) and receive updates through their mailing list.

46. Testing genetic therapy treatments for epilepsy

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

Gene Therapy, Epilepsy, Efficacy, Tolerability, Genetic 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?

We have recently created a series of new treatments for severe epilepsies. The overall aim of this project is to show these treatments are safe and effective and ready to progress to first in human 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?

We are in a new phase of medical research, with gene therapy opening doors for treatments that can repair cells in our bodies in ways that were simply not possible a decade ago. Our group has been world-leaders in using careful animal studies to learn what has gone wrong in disease, and to harness the promise of genetic therapies for repairing these problems to treat disorders of the brain. In order to deliver the promise of new genetic therapies to the clinic, after we have developed potential treatments in cells and other non-animal models, we need to confirm the treatments are safe and effective in the best available models of diseases to be sure our treatments do not harm patients and have the best chance of curing their symptoms.

Our research has shown our new treatments are effective in laboratory conditions, but before regulators allow us to progress to treating patients, we need to carry out standard testing to confirm they are safe, and what doses are likely to be best to use in people. We cannot do these studies as normal research projects as they are focused on clinical translation of existing treatments, not identifying new treatments.

What outputs do you think you will see at the end of this project?

By the end of this project we hope to have safety and efficacy data that will support up to 6 new gene therapy treatments for epilepsy to progress to first in human clinical trials. Specifically, we will have tolerability data showing the new treatments do not cause damage to the brain at up to 3 doses, and we will have efficacy data showing for up to three doses whether or not the treatment is effective at reducing seizures at that dose without side effects on thinking or mood.

Who or what will benefit from these outputs, and how?

1. Our ultimate goal is to offer new hope to many thousands of patients with severe refractory epilepsy, who currently have no effective treatment. We are preparing clinical trials now (our first trial is registered on clinicaltrials.gov NCT04601974), and hope to be treating patients with our first gene therapies by the end of this project. (First in human trials are planned to begin in 2023).

2. Our first-in-human trials will aim to offer less invasive, and better tolerated treatments to patients approved for surgery to remove the part of their brain that is causing epilepsy.

3. In the longer term, we believe our treatments may also be applicable to other diseases and disorders that are associated with altered activity in specific regions of the brain, this includes chronic pain, depression, anxiety and addition, as well as other disorders

How will you look to maximise the outputs of this work?

We have already established a spinout company to maximise the ability of our team to bring our new treatments to first-in-human clinical trials. Our aim is that this company will develop a portfolio of genetic and gene therapy treatments that will allow us to give precision therapies to people with different types of epilepsy, and eventually, to people with other serious neurological disorders.

Several of us occupy international roles in leading bodies in the field (ILAE, the International League Against Epilepsy) allowing a platform to reach leaders in clinical and basic 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.

All of the treatments that we will be testing on this project have already been tested in cell lines, and tissue. Many will have been tested in human cells. Before progressing to this project we will have data showing these treatments are effective in reducing seizures in animal models of epilepsy. The next step is to make sure they are safe enough for use in humans, and to determine, to the best of our ability, the dose, or how much we need to give to patients to stop seizures without risking causing damage.

We need to do this in adult animals that experience seizures, and we have invested in creating mouse models of epilepsy, that are close replications of refractory epilepsy that we see in our clinics. To give the best chance of successful translation from mice to human, we try, where possible, to test treatments in more than one model of epilepsy, in order to get an understanding of how widely applicable (or narrowly specific) a treatment may be: Is it likely to work in patients with many different types of epilepsy? or is it best restricted to just one or two specific types.

In addition, there is growing appreciation from patients and carers that treating diseases such as epilepsy involves more than just dampening excess brain activity. We also need to ensure other symptoms of these diseases (known as co-morbidities) are safely and effectively treated by our interventions. For this we need to use adult animals that have the symptoms of the disease (e.g. seizures), and are able to carry out behaviours such as learning simple mazes, or exploring a new space, which can inform whether they are also experiencing anxiety or learning disabilities.

Typically, what will be done to an animal used in your project?

This projects aims are to determine if our treatments are safe and effective.

To test whether the treatments are safe, we will carry out a surgery to inject a dose of the treatment into a specific area of the brain. We will test different doses (typically 3 different doses) into different animals to be sure our treatments are save across a range of doses. We will allow the animals to recover, and after several weeks we will collect tissue and examine it to see if there are any signs that the tissue has been damaged by the treatment. These animals will not experience any seizures.

To test whether the treatments are effective, we will need to work with animals that do experience seizures, as our main measure of efficacy is whether seizures are reduced in a way that will be meaningful to patients with epilepsy. We also have additional measures of efficacy, as patients ask us whether our treatments are also likely to help with anxiety and learning challenges. For these studies, animals will be administered protocols that are known to induce seizures or seizure-like activity in the brain. Once the seizures are established the animals with be administered our treatment, and their brain activity will be recorded to see whether the seizures stop. We will also use a series of usually non-stressful behavioural studies to see if they are less anxious, and better able to learn after treatments. To be sure our treatments are long lasting we will follow animals for several weeks after treatment.

What are the expected impacts and/or adverse effects for the animals during your project?

We do all we can to minimise suffering, but in order to test the efficacy of our treatments against seizures, it is necessary that animals do experience the sorts of seizures we wish to treat in patients. Patients report that seizures are not painful, but that afterwards they are tired, and if they fall, they may be injured. Increasingly, we are able to develop treatments that reduce these seizures in animals over a few weeks, but our 'control' animals can continue to have seizures for the duration of the study, which is usually less than 12 weeks for mice and only occasionally longer (when we need to ensure that the treatment remains effective for a long time - sometimes up to 6 months). We make every effort to keep animals healthy and comfortable, but unfortunately epilepsy (in humans as well as in mice) is associated with SUDEP (Sudden Unexpected Death in Epilepsy). This means a small number of animals (~1-5%) die for reasons that are still not understood, but which are thought to be associated with the seizures.

The additional impact is that all animals will undergo aseptic surgery, and some will have two surgeries, as we need to induce epilepsy prior to treating it in order to better replicate the clinical scenario (patients do not come for treatment until after they have developed epilepsy!). Some animals do not react well to surgery, and we do occasionally see some weight loss. Weight loss is usually transient, and once animals recover, they put weight back on. Sometimes animals pick at their sutures as well, and can even re-open a sutured incision. Typically this sign of irritation and itching goes away after a few days, as the incision heals.

To measure the flinch of the animal upon the sound, the animals need to be enclosed in a small chamber that is placed on a very sensitive balance. Being in these small chambers can also induce stress in animals, as they have little place to move during the test. But these small chambers allow us to very accurately measure subtle movements in mice.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We expect overall the severity will be moderate in ~95% of animals (because they will all have surgery), and severe because of SUDEP or poor surgical recovery in ~5%.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Our main aim is to develop new treatments for neurological diseases. Much of our preliminary work is done in cells and cell lines, but ultimately in order to show the

treatments are effective on symptoms such as those experienced by patients with epilepsy, we need to work with animals that have these symptoms. Where possible we use human-derived cells, including iPSCs, to determine how diseases might affect single cells, but in order to understand seizures we need to use live animals that can experience seizures. We have worked, where possible, with models in fruit flies who share many genes with humans, but many of our treatments are at an advanced state of moving to first-in-human clinical trials, and before we are allowed to test these treatments in human patients we need to be convinced they work and are safe in mammals that share the same types of neurons in their brains as humans have.

Another important concern comes from patients and carers who are telling us that challenges with behaviour and memory are also very important to their well-being, and we are working to be able to test whether our treatments work to help behaviours such as memory and anxiety. We need to carry out studies in living animals, including animals with seizures or other symptoms in order to measure if our treatments can help with these behaviours.

Which non-animal alternatives did you consider for use in this project?

Before we test treatments in vivo, we use cell lines and cultured cells to screen our treatments. This includes, where possible, induced pluripotent stem cells (iPSCs) to validate our treatments as much as possible before moving to animal cells. Only treatments that have already passed all these hurdles are moved to this project.

Why were they not suitable?

We find cells and cell lines are suitable for understanding many aspects of how a treatment works, and we do a substantial amount of work to understand our treatments and the causes of disease in cells and cell lines before using any animals.

However, in order to test for whether a new treatment can stop a neurological disorder, we need to use animals that have the same types of neurons as humans do. Studies in cells or networks of cells grown in petri dishes can only assess whether our treatments work at the cellular level, or in simple networks - which is very useful for knowing whether to continue our studies of a treatment, but isn't enough information to risk injecting a new treatment into a patient. Ultimately in order to be confident enough to deliver what may be an invasive and permanent treatment to a patient's brain, we need to be very confident that the treatment is safe and effective, and testing in living, behaving animals is the only way we have to be confident that our treatment can stop symptoms that involve networks within 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?

All the treatments we are testing here will have already been validated in our laboratories. The extension here is to test additional doses. So we know that for each test of safety we need about 8 mice per dose. We also know from our studies of efficacy that to see robust, reproducible changes in seizure numbers, and improvements in behaviour we will need closer to 20 mice per dose. This means to test each new treatment, we will need approximately 80 mice (this includes controls). We are hoping over the course of 5 years to test approximately 10 treatments and are including a few additional animals to allow for cases where we need to test more than 3 doses.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

For all our new treatments we use a study design that is powered to give clinically meaningful effects with the smallest number of animals. Where possible we use animals as their own controls, as this can reduce the number of animals needed to see a meaningful effect. We are also pioneers in carrying out long lasting recordings of brain activity that allows us to collect much more data from each animal, and this reduces the number of animals needed to power our studies of efficacy.

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 the studies we plan here are informed by our research on our partner PPL, where we have extensive data indicating how the treatments work, and how variable they are. This allows us to carry out accurate power calculations for our efficacy studies.

We routinely carry out our studies in a blinded, randomised design so that we reduce bias. This means we are less likely to think a treatment is effective when it is not, and therefore less likely to keep testing it in additional models.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 worked with these models of neurological disorders for many years, and have refined them to reduce suffering and distress. Over the course of our studies we have refined surgeries, so that very few animals have adverse effects, and new surgeries are piloted in individual animals and then small groups to be sure the new methods are well-tolerated.

We collaborate widely in epilepsy research, and implement changes reported by any research groups in the field that refine our models. However, in order to show treatments are effective at reducing symptoms, it is unavoidable that animals do have to experience

the symptoms when untreated (we hope the animals that are treated will have many fewer or no symptoms, of course).

We are constantly updating our models of epilepsy to find those which are most tolerated by animals, but which are also most relevant to human disease so that we are most confident our new treatments will translate to human patients.

We do have multiple models of epilepsy, including some which are more serious. This is because there is a risk that if a treatment is only shown to be effective in a single model of epilepsy it may not translate to be an effective treatment in human patients. Therefore the gold standard in the field is to test treatments in more than one model of a disease, ideally with different mechanisms. We aim to show each of our treatments is effective in at least two different models.

Why can’t you use animals that are less sentient?

Where possible we do use neonatal animals to collect tissues to carry out studies in vitro. Only when we are confident our approaches work in vitro using these immature tissues do we progress to using mature animals. We also test in cell lines and human derived cells (iPSCs).

We collaborate with groups that use less sentient models, including slime molds and fruit flies, and in some cases have had very important insights into the mechanisms and treatments of diseases from these organisms. However many of the epilepsies we wish to treat are associated with brain structures that do not exist in these less sentient organisms, so they cannot be well-studied or treated in these organisms. Also some of our treatments target types of brain cells that don't exist in fish or fruitflies, so we cannot test them in these organisms.

Where possible we do use animals under terminal anaesthesia for some studies in our partner licence, prior to moving animals to this project. However anaesthesia interferes with brain activity, and the studies here rely upon knowing whether a treatment changes brain activity, so it is necessary to study the efficacy of these treatments in animals that are awake.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We regularly monitor animals after any surgical procedure to ensure they are recovering well. We provide wet food, and when animals show signs of weight loss we have a food pantry of treats to help encourage animals to eat (Nutella is a favourite).

We regularly consult with our NVS to ensure our pain management is as up-to-date and effective as possible.

Working with our technical staff we work to provide all animals with safe and fun enrichment, including running wheels (mice only), houses, tunnels and bedding.

Some animals with epilepsy or implanted devices need to be housed individually. We have worked to introduce periodic social sessions, and cages with transparent dividers so animals can interact with peers even whilst housed separately. Many animals with epilepsy

are monitored with 24/7 videoing as well as EEG, which can indicate if number of seizures is increasing, or if individual seizures are prolonged.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Local NVS and NACWO are our first points of contact for queries. We also use resources hosted on the NC3Rs website, in particular:

- ARRIVE guidelines on experimental design and reporting results.
- 'Procedures with Care': 'Aseptic Technique in Rodent Surgery'.
- Rodent housing and husbandry
- Mouse Grimace scales

For pre-clinical studies we follow guidance given by the MHRA on animal numbers and time points.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The PPLh is the Chair of the local AWERB which has representation by the NC3Rs representative. The AWERB has a regular and strong emphasis on collecting and sharing best practices from across the

institution and elsewhere, with an aim of identifying and promoting any new approaches that advance 3Rs in any area of research.

47. Role of galectins in the regulation of chronic inflammation

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

fibrosis, cancer, therapeutics, galectins, inflammation

Animal types	Life stages
Mice	adult, pregnant, juvenile, neonate, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to gain a better understanding of the role of galectins and LOX-L2 in controlling aspects of cell behaviour associated with the development of inflammation, fibrosis and cancer and to develop novel therapeutics.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Galectins are central regulators of inflammation and fibrosis and have been shown to predict disease severity and outcomes in several fibrotic diseases and cancer. LOX-L2 is a key enzyme regulating collagen production and scar formation. The work outlined in this application identify the roles of specific galectins (1, 3 and 9) and LOX-L2 in disease processes and will direct the development of novel inhibitors to treat those diseases in which they play an important role in particular acute lung injury (ALI), pulmonary fibrosis, liver and cardiac fibrosis and cancer which have a large unmet clinical need.

Idiopathic pulmonary fibrosis (IPF): IPF is an ultimately fatal disease with a median survival from the time of diagnosis of 2.9–5.0 years. There are approximately 5,000 cases of IPF diagnosed in the UK each year, and the incidence is rising. There are two approved products for the treatment of IPF in the EU: Esbriet (pirfenidone) and Ofev (nintedanib) which have limited efficacy and significant side effects which lead to discontinuation in around 50% of patients. With no other therapy available there is a large unmet medical need for a targeted therapy with a clear mechanism of action that could halt and potentially reverse disease progression with an improved side effect profile.

ALI: There remains no standardized treatment strategy for the management of ALI in any context. Current treatment consists of palliative care (oxygen administration, hydration) steroids and antibiotics. For chronic disease the 2 anti-fibrotic therapies - pirfenidone and nintedanib – have yet to demonstrate efficacy to stop or reverse lung fibrosis and will present with the same tolerability issues as with IPF. New strategies are therefore urgently required for the treatment of chronic disease, with the goals of resolving lung damage and ameliorating scarring and obstruction.

Liver Disease: Liver disease is a global epidemic and a very large unmet medical need. 1/4 people in the world have fatty liver disease and it is estimated that 20 million of those will die liver-related deaths. Type-2 diabetes is strongly associated with non-alcoholic liver disease (NAFLD) and the obesity crisis could result in a trebling during the next 20 years to 6.2 million by 2034. Continued inflammation and fibrosis is a feature of liver disease regardless of etiology and progressive fibrosis eventually evolves to cirrhosis. No anti fibrotic drugs are currently licensed by the FDA for use in liver fibrosis, which has been designated orphan disease status. The global annual market for liver disease could be as high as $40 billion by 2025. Galectins have a key role in the progression of NAFLD and NASH and galectin- 3 is used as a histological biomarker of disease progression.

Heart Fibrosis. An estimated 1 million people in the UK are currently living with myocardial infarction associated conditions and over half a million with heart failure. Although treatment for heart failure has improved significantly there is still a need for new approaches to prevent its development. Galectin-3 is a marker of disease severity and can predict poor outcomes in patients with heart failure. Targeted inhibition of galectin-3 would provide a novel and safe approach to treat cardiac fibrosis.

Cancer: Cancer affects 1:4 people at some stage during their liftetime. There are around 375,000 new cancer cases in the UK alone every year and around 167,000 cancer deaths. Only around half of people diagnosed with cancer survive their disease for ten years or more. New and less toxic therapies are urgently required particularly in resistant and advanced disease. The main therapy for most cancers is a combination of chemotherpy and/or radio therapy which carry a significant side effect profile and inevitable lead to tumour resistance. More recently immune checkpoint inhibition (CPI) is undergoing extensive clinical investigation in non-small cell lung cancer (NSCLC) and other solid tumours via the use of the anti-CTLA-4 antibody ipilimumab and several targeting PD-1 (nivolumab, pembrolizumab) and PD-L1 eg atezolizumab. However accumulated data from clinical trials has suggested that the anti-tumour response rate of immune checkpoint inhibitors is not as high as was initially expected due to T cell exhaustion. Special focus is being directed at increasing T-cell infiltration into tumors, thereby increasing the effects of immune checkpoint inhibition. Data generated from my current PPL has shown that galectin-3 in the tumour microenvironment induces resistance to CPI and that treatment with galectin-3 inhibitors restores sensitivity to CPI inhibitors. Consequently the

galectin-3 inhibitor GB1211 is in clinical development in patients with advanced NSCLC in combination with atezolizumab (Gallant-1 NCT05240131) and about to commence a clinical trial in combination with pembrolizumab in advanced head and neck cancer and melanoma.

What outputs do you think you will see at the end of this project?

Publications - in peer reviewed journals

Presentations at national and international conferences

Patents filed for novel compounds

Identification of candidate compounds expected to be progressed to clinical trials

Who or what will benefit from these outputs, and how?

This project is aimed at understanding the role of galectins and LOX-L2 in the development and therapy of inflammation, fibrotic disease and cancer. The outcomes of this work will direct the development of novel inhibitors to treat those diseases in which galectins and LOX-L2 play an important role in particular acute lung injury (ALI), idiopathic pulmonary fibrosis (IPF), liver and heart fibrosis and cancer, diseases which have a large unmet clinical need.

Short term Benefits:

In the short term this project will lead to further elucidation on the mechanisms of action of galectins (1, 3 and 9) and LOX-L2 in diverse models of inflammation, fibrosis and cancer. It may also implicate galectins as biomarkers of disease severity, thereby identifying individuals or subgroups of individuals with a greater potential to benefit from anti-galectin therapy. The work will also benefit other scientists working in this area in the near term allowing them to focus on the key fibrotic pathways influenced by galectins and LOX-L2. We will publish and disseminate our data via conferences and publications.

Medium Term Benefits:

The work in this application will guide further clinical development of galectin and LOX-L2 inhibitors and identify new novel and specific inhibitors into new therapeutic areas.

Long Term Benefits:

The clinical development of galectin and LOX-L2 inhibitors will open up an exciting, new therapeutic avenues in the search for effective treatments for acute and chronic lung injury, fibrosis and cancer. As an inhaled galectin targeted therapy has been shown to be safe we would expect an easy transition towards its development for other respiratory indications. We believe this approach will be beneficial both in terms of patient wellbeing and compliance. With the developing understanding of other galectin family members in fibrosis and cancer and the large database of current and new inhibitors with improved selectivity and potency our aim will be to develop other novel compounds for use in clinical studies.

How will you look to maximise the outputs of this work?

The data will be presented to the clinical project teams and provide essential information for further clinical development of novel inhibitors

The data will be presented at scientific conferences and published in open access journals

Species and numbers of animals expected to be used

- Mice: 8000 mice comprised of 5000 commercially available mouse strains and 3000 GA mice including breeding

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 only adult mice and the procedures are well validated with defined endpoints. We have chosen mice as some of the work will be compared to mice that have genetic alterations which have had galectin genes either deleted or replaced with human galectin-3. The protocols are designed

to be as short as possible to minimise discomfort whilst inducing a relevant injury response that can be assessed using well validated assays.

Typically, what will be done to an animal used in your project?

This project includes breeding of genetically altered (GA) animals for use in this study (Protocol 1).

Animals will be ear notched for identification. Offspring generated from this protocol will be kept for continued use on protocols 2-8. Animals may be used for natural mating on a number of occasions. Animals produced under this protocol are not expected to exhibit any harmful phenotype. Offspring will be maintained until they reach a maximum of 15 months of age after which they will be killed by a schedule 1 method.

The experimental protocols in this project are designed to induce an inflammatory or fibrotic injury in different organs or to induce tumour growth.

Inflammatory procedures (Protocols 2-4):

Inflammation will be induced by a single administration of an injurious agent either by intratracheal administration or injection.

Administration of LPS systemically - Protocol 2

Administration of LPS intratracheally - Protocol 3

Administration of concanavalin-A or paracetamol or CCl4 -Protocol 4
These procedures are short and will typically be monitored over no more than 72h. In some cases, mice will be given test substances either prior to or daily by one or two enteral or parenteral routes for the duration of the study. In some instances therapeutic

intervention may be delayed to observe effects on resolution of inflammation. Blood samples will be taken, typically three times from a peripheral vein and some animals may receive BrDU or cell labelling agent to assess cell proliferation up to 2h prior to cull. At the end of the study animals will be killed by a schedule 1 method or by exsanguination under terminal anaesthesia.

Fibrotic procedures (Protocols 5-7)

Fibrotic injuries are designed to induce an inflammatory response followed by progression to fibrosis. In these procedures the animals recover quickly from the initial inflammation and will be monitored during the chronic phase.

Induction of lung fibrosis - Protocol 5

Mice will be administered bleomycin or silica or saline control either intra-tracheally or intra-nasally by either a single administration or from up to 3 divided doses. A subset of animals may recieve an additional single administration of LPS (or saline control) intra-tracheally at 14 - 21 days to model an acute exacerbation of IPF. In some cases, mice will be given test substances or control either prior to or after the start of study by one or two enteral or parenteral routes. Animals will be monitored for up to

60 days or in the case of aditional LPS administration no more than 2 days post LPS. Blood samples will be taken, typically three times from a peripheral vein. Some animals may receive BrDU or cell labelling agent to assess cell proliferation up to 2h prior to cull. Some aninmals may undergo non invasive in vivo imaging (MRI or optical molecular imaging). Imaging procedures may include the intravenous or intraperitoneal injection of contrast agents. At the end of the study animals will be culled by a schedule 1 method or by exganguination under terminal anaesthesia.

Induction of liver fibrosis - Protocol 6

Liver fibrosis will be induced by 1 of the following means Induction of liver injury using CCl4

Mice will receive CCl4 diluted in sterile oil for up to 16 weeks administered up to thrice weekly by intra- peritoneal injection or orally by gavage. Sterile oil will be used as a control. If observing resolution of fibrosis the animals may be observed for up to 3 months following the final dose of CCl4.

Induction of non-alcoholic steato-hepatitis (NASH) and liver fibrosis by Administration of choline deficient amino acid defined high fat diet (CDAHFD) diet for up to 12 weeks or Administration of high fat high fructose diet plus CCl4

Mice will receive a high fat high fructose "western" diet typically containing 21.1% fat, 41% sucrose, and 1.25% cholesterol plus a high sugar solution (23.1g/L d-fructose and 18.9 g/L d-glucose) in the drinking water for up to 24 weeks. Mice will receive CCl4 at a low dose of 0.2 µl g administered intra- peritoneally once per week (or sterile oil control) starting simultaneously with the diet administration.
During the study the animals may receive therapeutic intervention or control by one or two enteral or parenteral routes. Blood samples will be taken, typically three times from a peripheral vein. Some animals may receive BrDU or cell labelling agent to assess cell proliferation up to 2h prior to cull. Some animals may undergo non invasive imaging on 1

or more occasions. Imaging procedures may include the intravenous or intraperitoneal injection of contrast agents. At the end of the study animals will be killed by a schedule 1 method or by exsanguination under terminal anaesthesia.

Induction of cardiac fibrosis - Protocol 7

Mice will receive angiotensin II by subcutaneous implanted osmotic minipump set to deliver up to 2mg/kg/day for up to 28 days. In most cases, mice will be given test substances or control by one or two enteral or parenteral routes. Control animals may not receive any substance administration. The majority of animals will undergo imaging up to 3 times during the duration of the study. Imaging procedures may include the intravenous or intraperitoneal injection of contrast agents. Blood samples will be taken, typically three times from a peripheral vein. Some animals may receive BrDU or cell labelling agent to assess cell proliferation up to 2h prior to cull. At the end of the study animals will be culled by a shedule 1 method or by exsanguination under terminal anaesthesia.

Induction of tumour growth Protocol 8

Tumour growth will be instigated by injection of tumour cells. Cells can be mouse strain matched wild type (syngeneic) or genetically modified tumour cells or human cancer cells (xenograft) administered to immunocompromised mice and may be accompanied by other components (e.g. extracellular matrix components/matrigel). Cells will be administered either subcutaneously into the flanks or into the mammary fat pad or by intravenous administration.

For subcutaneous injection into the flank or the mammary fat pad. In these animals hair will be removed around the the injection site by shaving or using hair removal cream and the area cleaned thoroughly with antiseptic.

Tumours will be monitored by the use of calipers in the case of subcutaneous tumours or by closely monitoring clinical signs and in some cases by in vivo imaging. The end of the experiment will be triggered when tumour volumes reach maximum allowed tumour volume or earlier. Typically this will be around 21 days for syngeneic models and 50 days for xenograft models.

A minority (<10%) of immunocompromised mice may be injected with human peripheral blood mononuclear cells (PBMCs) intravenously up to 1 week prior to or up to 3 weeks post human cancer cell innoculation to reconstitute a human immune system.

The majority of mice will receive a therapeutic regimen which may be a combination of galectin or LOX-L2 inhibitor plus immune checkpoint therapy or other anti-cancer therapy. Tumour volume of subcutaneous tumours will be monitored by caliper measurement. Some animals will undergo non- invasive In vivo imaging one 1 or more occasions. Imaging procedures may include the intravenous or intraperitoneal injection of contrast agents. Most animals will undergo blood sampling collected from a superficial vein on one or more occasion. At the end of the study animals will be killed by exsanguination under terminal anaesthesia or by a schedule 1 method.

What are the expected impacts and/or adverse effects for the animals during your project?

Typically, animals will show some clinical signs due to the administration of an inflammatory agent. This may be manifest as reduced mobility, loss of condition and weight loss of up to 20% - defined as moderate severity. The protocols are designed to be of short duration and not to exceed moderate signs of severity. In tumour protocols the tumour cells are injected in an area that would not affect normal activity (for example under the skin on the flank or in the mammary fat pad).

Mice may experience mild, transient pain but no lasting harm from administration of substances by injection using standard routes (intravenous, subcutaneous, intraperitoneal). Some animals will receive treatments by oral gavage. This should only result in mild transient discomfort. Flexible gavage needles will be used the minimise any injury to the oesophagus from prolonged dosing and gavage volumes will be small and will use vehicles that are nontoxic and which we have used previously.
Where administration is required for prolonged periods, animals may be surgically implanted with slow release devices such as a mini-pump. 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. 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. In animals injected with tumour cells the size of the tumour will be monitored using digital callipers or by imaging and animals will be taken off the study and humanely killed if the tumour volumes reach a predefined criteria. 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.

Expected severity categories and the 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 Protocol 1

Subthreshold = 90% Mild = 10%

Inflammation procedures (Protocols 2-4)

Subthreshold 0%, mild 25%, moderate 75%

Fibrosis procedures

Subthreshold 0%, mild 25%, moderate 75%

Cancer procedures 100% moderate

Subthreshold 0%, mild 0%, moderate 100%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Many of the experiments in this project will continue to use human cells (from volunteers and patients) wherever possible in order to, as far as possible, assess the relevance to human disease. These studies will not only guide the number and experimental design of the animal work but will replace the mouse usage where possible. It is not feasible to produce an adequate in vitro or in silico model of the immune system and the body's response to infection, thus in this regard there is no substitute to live animal studies to determine systemic responses to novel treatments. Cancers are very complex, and involve a number of different cell types e.g. epithelial cells, immune cells, blood vessels, all of which can contribute galectins to the tumour microenvironment. As yet no cell culture system exists that faithfully recapitulates this environment. The involvement of the tumour microenvironment and the immune system on cancer cell development and response to therapy is well documented and highlights the need to use mouse models for these studies.

Which non-animal alternatives did you consider for use in this project?

We have looked at using human tissue slices and human tumoroid systems.

I have searched FRAME http://www.frame.org.uk/ and the website Norecopa https://norecopa.no/ for suitable in vitro alternatives.

I have reviewed the European Commission's Joint Research Centre (JRC) published review of advanced non-animal models being used for basic and applied research into respiratory tract diseases and the nat-database.org website

Why were they not suitable?

Despite being extremely costly these systems do not accurately depict an ongoing fibrotic event or tumour development as they lack the involvement of the immune system in sculpting this response. Even though immune cells can be added in those systems it does not accurately predict how the injured site may recruit cells in vivo. As galectins interact between the innate and adaptive immune system this is difficult to model 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 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.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In order to optimise our experiments, we will use previous publications and pilot experiments already performed. In drug intervention studies small dose finding studies using small numbers of mice (n=3) will be used to accurately optimise the dose required for further use in experimental protocols.

The use of non-invasive in vivo imaging (e.g. ultrasound, fluorescence) to detect and measure internal tumours allows us to carry out longitudinal studies. This greatly reduces the number of mice required, as we do not need to euthanize separate cohorts of mice at different time points to assess tumour burden.

The applicant is fully aware of the Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines and the principles guiding the Replacement, Refinement and Reduction of Animals in Research (www.nc3rs.org.uk/ARRIVE/). All experiments will be executed adhering to the ARRIVE guidelines and the principles governing the NC3Rs to the best of the applicants' ability. The applicant has also consulted the Planning Research and Experimental Procedures on Animals: Recommendations for Excellence (PREPARE) guidelines to ensure quality and optimise reproducibility of the experimental protocols. With particular reference to our newly created transgenic animal lines, all wild-type litter mates (approx. 50%) generated throughout the breeding phase, will be used as our age matched control animal groups, thereby eliminating the need to obtain wild-type animals for this purpose and reducing unnecessary cost.

"We also have access to the NC3rs experimental design assistant resource which may be helpful for experimental design of studies:https://www.nc3rs.org.uk/experimental-design-assistant-eda ."

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

All of the experimental protocols in the programme have well-validated and robust pre determined end- points, while local expertise and experience in each of the experimental protocols means that the expected spread of results for key end-points are already established. We will also maximise the number of readouts assessed per animal thus reducing animal numbers required. Tissues that are not immediately analysed will be stored either frozen or fixed for downstream analysis and may be made available to other investigators working in this area.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We need to monitor an inflammatory response in mice to effectively assess the effect of galectin inhibition. We have chosen well described models of acute inflammation, (systemic, lung and liver) and have refined the endpoints to shorten the duration and minimise dose of inflammatory agent. Frequent monitoring and clinical score sheets will ensure no animal suffers unduly.

In the fibrotic models longer study times are necessary to mimic human disease, however again our previous expertise with these models allow us to pick the shortest meaningful timepoint to assess effect of treatments.

The model of high fat feeding in mice is longer - up to 24 weeks. This is necessary to mimic human non alcoholic steatohepatitis (NASH) which is a protracted chronic disease. In some cases a low dose CCl4 will be used to accelerate fibrosis in this model and lead to early hepatocellular carcinoma (HCC) development. There are no shorter models of HCC that have been well described in the literature.

The tumour models used in this project are either syngeneic ie transplantable mouse cancers growing in mice, or xenografts ie human cancer cells growing in immunocompromised mice. We have expertise in both of these models. The former is generally of shorter duration (2-4 weeks) and involves all aspects of the immune system. The latter is longer (4-6 weeks) and although uses human cancer lines the animals have no adaptive immunity. In a small percentage (10%) of studies we aim to transplant human peripheral blood monocytic cells (PBMCs) as a means of recapitulating the human immune response.

We have generated GA mice that either express no galectin-3 or the human galectin-3 gene, which will provide a more directly translatable model for human disease.

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 or very young animals as their immune system is immature and doesn’t respond to antigenic stimulation in the way mature animals do.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have refined our experiments using intratracheal instillation. We use a blunted catheter applied between the vocal cords under direct trans illumination of the thorax, thus obviating the requirement for the traditional approach of surgical exposure of the trachea. For repeated administration of therapeutics we use the oropharyngeal route of administration which uses very light and brief anaesthesia.

- Each of the experimental protocols in this proposal incorporates monitoring of animals both by ourselves and by experienced staff in the animal facilities. These checks

are in place such that unexpected suffering is rapidly assessed and alleviated using analgesia, anaesthetic or humane killing as appropriate. Good practice in the clinical assessment of pain and distress in rodents will be observed according to the FLAIRE learning emodule (recognition and prevention of pain, suffering and distress in lab animals).

- In most of the experimental protocols described adverse events can be expected to be established within a few hours. Therefore, wherever possible we shall time experimental protocols to begin as early as possible in the working day such that animals can be monitored frequently during the period in which they are most susceptible to adverse events.

- In all of our protocols we utilise validated humane endpoints, which trigger termination of a protocol. For example with the liver fibrosis dietary models there is some natural variability in how the mice respond to the diet in terms of weight loss, we therefore set a limit of percentage weight loss in agreement with the NVS below which animals cannot drop.

- Pilot studies form an important part of the programme such that the minimum dose of an inflammatory agent reproducibly causing the appropriate pathological effect is used. This approach will substantially reduce the risk that experiments prove interpretable because of unexpected results.

- The orthotopic tumour models have been chosen to address different aspects of cancer progression e.g. tumour growth, metastasis to distant sites such as lung. Use of immune- compromised mice for these studies is the only way in which we can monitor behaviour of human derived tumour material. The use of non-invasive in vivo imaging allows us to detect internal tumours at an earlier stage of disease progression and reduces the need for intrusive procedures.

- Transplantation of human cancer cells (xenografts) into immune compromised mice is a widely used technique which typically does not result in any adverse effects. However timour growth will be closely monitored to ensure timours do not ulcerate or not impair mobility. Should animals experience unexpected metastasis, or difficulty feeding or moving due to the tumour burden or demonstrating ulceration will be terminated via schedule 1

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We have consulted the Planning Research and Experimental Procedures on Animals: Recommendations for Excellence (PREPARE) guidelines to improve the quality reproducibility of our studies

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will regularly check information on NC3Rs website and attend Regional 3Rs symposia.

48. Filling data gaps for elasmobranch conservation in Wales

Project duration

5 years 0 months

Project purpose

- Research aimed at preserving the species of animal subjected to regulated procedures as part of the programme of work

Key words

Elasmobranchs, conservation, movement, habitat use, Wales

Animal types	Life stages
Angelshark (Squatina squatina)	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 fill critical data gaps on the distribution, ecology and movement of threatened elasmobranchs (sharks, skates and rays) in Wales and surrounding waters, to inform conservation and management 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?

The marine environment in Wales supports diverse fish communities including little-studied species of shark, skate and ray (elasmobranchs) of conservation importance. Elasmobranchs are one of the most highly threatened taxonomic families (Stein et al., 2018) with over one-third of species thought to be threatened with extinction (Dulvy et al., 2021). Globally, targeted and incidental catch in fisheries are the primary threat to these species, with many particularly vulnerable due to their life history, e.g. low reproductive output and slow growth rates (Dulvy et al., 2014). In addition, species that utilise coastal areas or spend the majority of time close to or on the seafloor (benthic or benthopelagic),

are further at risk due to habitat loss and modification caused by a range of direct and indirect pathways (e.g. marine developments).

In total, 27 coastal elasmobranch species are found in Wales, but research has mainly focused on species with the greatest commercial importance to inform stock assessments (e.g. thornback ray). The absence of robust baseline data on the biology and ecology of many elasmobranch species in Wales, including potential links with protected habitat features in Special Areas of Conservation (SAC), means that it is challenging to make accurate assessments about the health, structure and function of these ecosystems to feed into conservation and policy decisions. This programme of work focuses on using telemetry and genetics to answer key research questions identified from data gathered over the last six years via fisher local ecological knowledge and non-invasive surveys (e.g. surveys using environmental DNA and Baited Remote Underwater Video Systems). This is particularly pertinent as 18 of the elasmobranch species found in Wales are listed in a threat category on the International Union for Conservation of Nature (IUCN) Red List of Threatened Species global assessments.

This programme of work is part of Project SIARC (Sharks Inspiring Action and Research with Communities), a multidisciplinary project with several partners led by ZSL and Natural Resources Wales, which integrates biological, engineering and social sciences. We focus on elasmobranch species of conservation importance to maximise the benefits of conducting research, i.e. focal species that are data limited, benthic or bentho-pelagic, listed in a category of threat on the IUCN Red List of Threatened Species, protected by national or international legislation and recommended for inclusion by fishers involved in the project to date. Our collaborative approach enables the outputs of this work to have a long-lasting benefit for policy, conservation, science, welfare and communities local to our project sites.

What outputs do you think you will see at the end of this project?

Policy outputs:

Updated Project SIARC Recommendation Document, which will use results of the programme of work to provide key recommendations for public bodies, competent authorities, consultants, agencies, developers and other relevant groups in Wales when considering the potential impacts of activities and developments in the marine environment, in relation to elasmobranchs of conservation importance.

Data gathered from the programme of work will be synthesised into data layers in a variety of formats and uploaded onto national databases (e.g. Data Map Wales, NBN Gateway, Fishbase) to enable utilisation by relevant parties.

Conservation outputs:

Data will be incorporated into an updated Wales Angelshark Action Plan planned for 2025, which will highlight conservation and research priorities for angelsharks in Wales and identify where best to allocate resources.

A summary report will be developed, highlighting how elasmobranchs use the Pen Llyn A'r Sarnau Special Area of Conservation (SAC), to support decision-making in the SAC.

Scientific outputs:

At least twelve months of fine-scale elasmobranch movement behaviour in North Cardigan Bay (Wales) recorded in an acoustic detection database.

Maximum likelihood tracks generated to evaluate broader elasmobranch movements, with vertical and thermal habitat utilisation quantified for elasmobranchs tagged with miniPAT tags.

Elasmobranch tissue samples collected for genetic analysis, sequenced and compared with population(s) outside of Wales to assess connectivity.

At least three scientific manuscripts submitted for peer review publication.

Welfare outputs:

Methodologies will be written up as best-practice guidance and shared with researchers working on the same species via the large scientific network.

One scientific manuscript submitted for peer review publication focused on methodological advances for angelshark.

Community outputs:

Results of the programme of work will be disseminated in bi-lingual outputs for local communities and will be distributed in a variety of ways: infographics, social media posts, presentations, public talks, school sessions and focus group discussions.

Who or what will benefit from these outputs, and how?

Long term benefits (>5 years)

Ultimately, data collected as part of this programme of work will be used to inform evidence-based conservation and management decisions to preserve the species and improve the conservation status of elasmobranchs in Welsh waters over the long-term. There are several other benefits in the short- term, as outlined below:

Policy outputs:

The policy outputs will be primarily focused for use by Natural Resources Wales (NRW) Marine Area Management Teams and Marine Licensing Teams, who are responsible for assessing marine activities and developments in Wales, to ensure that elasmobranchs are considered when potential impacts on the marine environment are being formally assessed. This includes providing advice on licensable marine activities (any deposit or removal of material or substance using a vehicle or vessel; construction, alteration or improvement works; scuttling vessels or floating containers; dredging; or incineration of objects or use of explosives); non-licensable marine activities (dredging and disposal authorised under local Acts or Harbour orders, activities related to recreational boating, navigational markers); and review of Environmental Impact Assessments for developments that might have an impact on marine ecosystems.
These NRW teams are also involved in relevant national legislative and policy update mechanisms alongside Welsh Government; data from this programme of work will also be considered, where appropriate, in review of Section 7 of the Environment (Wales) Act, Schedule 5 of the Wildlife and Countryside Act (1981) and the UK Fisheries Act (2020). It will also support NRW and Welsh Government when reporting on international protections

for elasmobranchs (OSPAR List of Threatened and/or declining species and habitats, Convention on the Conservation of Migratory Species of Wild Animals (CMS), Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), and United Nations Convention on the Law of the Sea (UNCLOS)).

Outputs will also be widely shared with a broad network of relevant parties, including public bodies, competent authorities, consultants, agencies, developers, technical specialists and other relevant groups in Wales who may be involved in marine activities or developments.

Conservation outputs:

The Summary Report will be carefully co-designed with SAC Officers and NRW staff to link with the Section 37 Conservation Objectives Advice package updates, so that elasmobranchs can be included in site management for Pen Llyn A'r Sarnau SAC, where relevant. The Action Plan will be developed using a participatory approach with representation from government, research, conservation, fishers, water users and local communities. Once finalised it will be shared across Project SIARC's broad spectrum of contacts.

Scientific outputs:

Project SIARC hosts a Research Group with over 50 partcipants, which provides a forum to facilitate dialogue and foster new collaborations on elasmobranch research in Wales. All outputs will be shared with the Research Group and support the sharing of knowledge and expertise, to increase research and conservation efficiency, avoid duplication and maximise impact of the work.

Publication of results in peer reviewed journals will showcase the work with a wider scientific community to benefit and foster further study on threatened elasmobranchs across their range.

Welfare outputs:

These will be shared across Project SIARC contacts, in particular with the Research Group where a meeting focused on animal welfare will be organised to share methodological information and best practices. The project team will also reach out to researchers working on the same species in other areas to discuss and share refinements used to improve elasmobranch welfare during the tagging process.

Community outputs:

Broad-based public support is essential for the long-term success of conservation efforts, as people who do not feel connected to nature will not feel motivated or empowered to protect it. Outside of this programme of work, Project SIARC has dedicated workpackages focusing on enabling community involvement and showcasing Wales' marine environment. Outputs of this project will be integrated within our wider Project SIARC communications strategy to achieve our aim that a greater diversity of communities in Wales learn about the marine environment, using elasmobranchs as a flagship in bilingual communications.

How will you look to maximise the outputs of this work?

Outputs will be bilingual (Welsh/English) for inclusive reach across Wales, the UK and further. Project outputs will be amplified through integration within successful Project SIARC communication lines to reach a wide public audience: sharing quarterly reports to relevant groups; completing interviews for local and national media; regular social media posts; website updates; other press releases at key outputs (recent BBC Wales coverage on 02/01/23 reached 2.6m people); and attending Wales-based events.

In addition, research and welfare outputs will be shared with a wider scientific audience through presentations at conferences and ensuring scientific publications will be Green-Open access.

Throughout the programme of work, we will closely collaborate with recreational, charter and commercial fishers in Wales, as their boats will be chartered to complete elasmobranch tagging. Regular meetings, feedback events, attendance at association/club events or AGMs, and focus group discussions will be used to keep fishers up to date on project progress, gather feedback on outputs to ensure key messages are representative of fisher experiences, and enable fishers to co-design how their data are used for policy and legislation. The role of fishers will be widely communicated, reinforcing their vital contribution to safeguarding elasmobranchs in Wales.

Species and numbers of animals expected to be used

- Other fish: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We have focused on elasmobranch species that are data limited, benthic or bentho-pelagic (i.e. spending time closely associated or on the seabed), listed in a category of threat on the IUCN Red List of Threatened Species, protected by national or international legislation and recommended for inclusion by fishers involved in the project to maximise the benefits of conducting research. These species have the most critical data gaps in Wales, which urgently need to be filled to inform conservation and management decisions.

The lifestages are selected based on where the need to fill knowledge gaps is most pressing and what is achievable given the rarity of the species.
Typically, what will be done to an animal used in your project? Source:
There are four different types of source within this project:

Source 1: Surveys will be completed with a charter boat skipper, experienced in best practice handling of elasmobranchs. Up to six rods with heavy duty traces and circle hooks baited with mackerel will be fished using a method and at a location to target angelsharks.

Source 2: Surveys will be completed with a charter boat skipper, experienced in best practice handling of elasmobranchs. Up to six rods with heavy duty traces and circle hooks baited with mackerel will be fished using a method and at a location to target tope.

Source 3: Opportunistic encounter of angelshark during diving or snorkel surveys.

Source 4: Attendance on a normal charter boat trip for tope.

Handling/Restraint:

Once an elasmobranch has taken the bait, it is reeled in and guided to the stern door or side of the vessel by holding the trace. Duration up to 8 minutes depending on depth.

For angelsharks: A landing net or sling is used to bring the angelshark on board, it is placed on a foam mat, the hook is removed, a damp cloth is placed over its eyes and it is restrained by two people when required (one holding pectoral fins, the other holding the tail). Water will be continuously pumped over the gills and the shark will be kept wet. Duration less than 5 minutes.

For tope: If sea conditions allow, the tope is restrained in the water on the side of the boat by the tail (tail-rope), mouth (hook) and body (canvas stretcher or strops depending on access to the animal from the boat); it is rolled over to be placed in tonic immobility. Duration less than 2 minutes. If sea conditions are too rough to appropriately handle the tope on the side of the boat, a landing net or sling is used to bring the tope on board, it is placed on a bespoke V-shaped foam holder, the hook is removed, a damp cloth is placed over its eyes and it is restrained by up to two people if required (one holding body, the other holding the top of the tail). Water will be continuously pumped over the gills and the shark will be kept wet. Duration less than 5 minutes.

Welfare and vitality assessment

A welfare and vitality assessment will be used to score clinical observations of appearance, body function, behaviour, and condition of the animal (Appendix 1 - image provided later in application). Scores will be used to assess whether the animal is in appropriate health/condition to tag. Length measurements and sex will be recorded during these assessments. Duration less than 2 minutes.

Multi-step procedure

The procedures used depends on the source of the animals. All animals encountered through Source 1 (n = 12) will go through Procedure 1, Procedure 3 and Procedure 4, with some (n = 3) of these also going through Procedure 2 (optional step). All animals encountered through Source 2 (n=30) will go through Procedure 1, Procedure 3 and Procedure 4. All animals encountered through Source 3 (n = 6) or Source 4 (n = 30) will go through Procedure 4 only. Flowchart is provided in Appendix 2 (image copied below).

Procedure 1 - external attachment of miniPAT satellite tag:

For angelsharks: A miniPAT tag will already be attached to an external ST1 button sure-tag; an incision is made using a drill onto the anterior margin of the left pelvic fin and the ST1 button sure-tag is applied using the tag applicator at that location. Duration less than 2 minutes.

For tope: a MiniPAT tag will already be anchored onto a titanium barb; a tag applicator similar to a handheld spear is used to implant the tag into the dorsal skin next to the dorsal fin. Duration less than 1 minute.

Procedure 2 – external attachment of innovasea acoustic tag (optional step)

For angelsharks: An acoustic tag within a bespoke tag holder is attached to the base of the first dorsal fin using a modified Allflex metal-tipped pin and tag applicator. Duration less than 1 minute.

For tope: not applicable

Procedure 3 – external placement of visual ID T-bar anchor tag (non-regulated as this is completed solely for identification purposes so as to prevent inadvertent re-use of animals if encountered during future surveys)

A T-Bar anchor tag with 6 cm long tubing with a 1.5 cm x 0.5 cm oval attached to the end is applied at a 45° angle into the dorsal musculature of the tail (angelshark) or body (tope) just below the first dorsal fin, using a hand-operated tagging applicator. Duration less than 1 minute.

Procedure 4 – tissue sample

A small tissue sample will be obtained from the tip of the second dorsal fin using sterilised surgical scissors. Duration less than 1 minute.

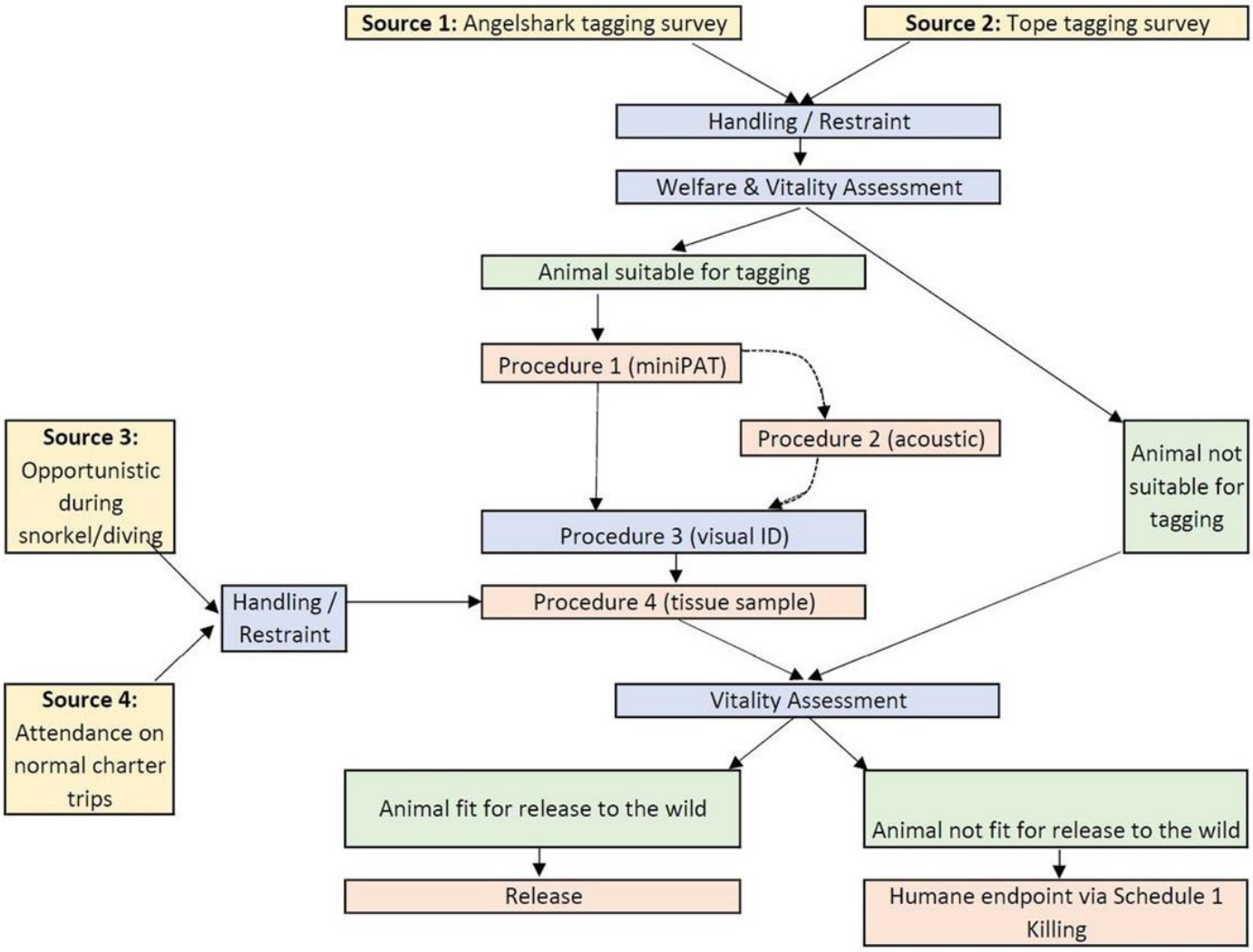
Vitality assessment and release to the wild.

A vitality assessment is used to assess the condition of the elasmobranch and whether it is fit for release to the wild. For angelshark or tope onboard the boat, they will be carefully handled back into the water through the stern door against the current. For tope aside the boat, the hook will be removed, the shark rolled out of tonic immobility and released against the current once it has retained full motion. Duration less than 1 minute.

Humane endpoint (if required)

If the welfare and vitality assessment score completed before the procedure or after the procedure shows that the animal is not fit for release to the wild, it will be euthanised using the Schedule 1 method “Concussion of the brain by striking the cranium”. This will involve a forceful blow to the back of the skull before being bled and is confirmed through destruction of the brain. This is a standard procedure for the euthanasia of large fish species. Duration less than 1 minute.

Appendix 2:



What are the expected impacts and/or adverse effects for the animals during your project?

This project will cause stress to all elasmobranchs involved (n=78) during the capture and restraint process as it will impact all five freedoms; in particular for adult angelsharks (n=12) or any tope that

need to be taken out of the water. However, this stress will be temporary, with a maximum protocol length of 22 minutes.

All elasmobranchs undergoing external tag attachment and/or taking of a small tissue sample are expected to experience minor pain of short duration (under 4 minutes) during the procedure.

A possible adverse effect could be infection of the tagging wound, but this will be minimised by using aseptic techniques throughout the procedure. A second possible adverse effect could be biofouling of the tag and tag attachment, but this is limited by the small size of the tags involved and the fact miniPAT tags release from the animals after a certain time. Another possible adverse effect is that tag attachment impacts animal behaviour, but this is expected to be negligible given the small size of tags involved and the careful choice and refinements made in tag design, location and methodology.

The degree of harm will be assessed by comparing vitality assessments before and after each procedure (Appendix 1 - image provided later in application).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Angelsharks (n = 18): 100% mild Tope (n=60): 100% mild

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?

The project is focused on filling critical data gaps in the distribution, ecology and movement of threatened elasmobranchs in Wales and surrounding waters. As such, we have to study the behaviour of wild-caught animals to be able to inform conservation and management decisions for the preservation of the species. Given that these species are so data-limited, especially in Wales, there are no complete or partial replacement strategies that can be used.

Which non-animal alternatives did you consider for use in this project?

A literature review was completed, but there are no non-animal alternatives as we are studying the behaviour of wild-caught animals to be able to inform conservation and management decisions for the preservation of the species.

Why were they not suitable?

There are no non-animal alternatives as we are studying the behaviour of wild-caught animals to be able to inform conservation and management decisions for the preservation of the species.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals used in this project is chosen based on pilot studies, published research and what is achievable in practical terms given the rarity of the species involved. Considered experimental design has been completed on the three inter-linked studies within the programme of work:

Angelshark telemetry

Research question: To what extent do adult angelsharks use the waters within and outside of the Welsh Zone during different seasons and what environmental variables are associated with this movement?

Null hypothesis: Adult angelsharks remain in shallow waters in the Welsh Zone year-round.

Alternate hypothesis: Adult angelsharks move offshore, into deeper waters, during winter months (October – March)

N = 12. The small sample size reflects the practical constraints of encountering angelsharks in Wales, given their rarity. Based on prior experience and the pilot survey, we estimate a maximum of 3 angelsharks encountered each year for a four-year period. However, the use of miniPAT satellite tags maximises the likelihood of gaining high quality and quantity data from each individual, thereby minimising the number needed to gain meaningful results. To further maximise data, three of these angelsharks will also be tagged with acoustic tags during the period acoustic receivers are deployed.

Type of study: correlational study

Type of experiment: exploratory study

Type of design: within-subject design

Dependent variable: temperature, pressure, light-levels (inferred location)

Independent variable / factors: sex, length

Randomisation design: Animals are randomly selected through fishing procedure (Source 1).

Blinding design: The person analysing the tag data will be provided coded information for sex, to minimise subjective interpretation of data.

Sex ratio: Our intention is to tag even sex-classes, but given that animals are randomly selected and miniPAT tags have a failure rate of 20-30%; the sex ratio is likely to be different to 1:1. We will tag any angelshark over 90cm that has been caught and assessed as appropriate to tag through the welfare and vitality assessments, to maximise outputs given the stress caused during the fishing procedure.

Statistical analysis: Analysis will follow that published in Curnick et al. 2020: location estimates will be processed using the miniPAT manufacturer's software, Geolocation Processing Estimator 3 (GPE3; Wildlife Computers Inc.,Redmond, WA, United States), which has been used widely to process elasmobranch tracking data. GPE3 generates two maximum likelihood position estimates per day using a hidden Markov model (Patterson et al., 2009) with a 0.25◦ × 0.25◦ grid spacing, and position estimates (two per day) interpolated and smoothed with a cubic spline. Program parameters will be defined for angelsharks to generate maximum likelihood position estimates and maximum likelihood tracks (MLT). Total track length (km) and displacement distance (kilometers between tagging location and pop-off location, km) will be calculated within the "move" package (Kranstauber and Smolla, 2015). The GPE3 generated 12-h probability density surfaces for each tag will be averaged and the 0.25◦ GPE3 grid resampled at a resolution of 0.0125◦ using bilinear interpolation using the R package "raster (sensu Stewart et al., 2016). From these, we will calculate the 50, 75, and 95% polygons as a metric for each animal's activity space from the merged probability surfaces and estimated their size (km2) and overlap (proportion of full probability surface) with habitat maps, protected sites and possible anthropogenic impacts (renewable energy, fishing effort etc.). Time series data will be split into diel phases using the R package "suncalc" (Thieurmel and Elmarhraoui, 2019) in combination with estimated daily locations, and mean depths occupied during the day and night will be compared using a Wilcoxon signed rank test for each individual. To minimise the influence of the capture process on shark behaviour, the first 24 h post-tagging from will be removed from analyses (Cliff and Thurman, 1984). In addition, analyses will explore how movement is correlated with sex, length and environmental variables (depth, temperature).

Pilot study completed in 2021: One pilot survey was conducted in collaboration with Cefas in July 2021, to test the bespoke tag attachment methodology that was designed during a series of workshops in 2021. One adult Angelshark was encountered during the 7 hours of fishing effort; it was tagged successfully with a miniPAT tag, a visual ID tag, and a tissue sample taken (total procedure lasted under 2 minutes), and was returned to the water in excellent body condition. Unfortunately, the miniPAT tag itself was part of a faulty batch and did not connect to satellite; so no data was recovered.

Examples of number of animals used in published research: Several published studies on satellite telemetry using animals with similar ecology to angelshark have generated sufficient data using small sample sizes:

Brewster et al. (2020) - Whitespotted eagle ray - 2 pSAT tags deployed (50% succesfully transmitted)
Francis et al. (2016) - Spinetail devilrays - 9 miniPAT tags deployed (78% succesfully transmitted)

Stewart et al. (2016) - Oceanic manta ray - 5 miniPAT tags deployed(80% succesfully transmitted)

Peklova et al. (2014) - Arctic skate - 9 miniPAT tags deployed (78% succesfully transmitted)

Le Port et al. (2008) - Short-tailed stingrays - 2 pSAT tags deployed(100% succesfully transmitted)

Tope telemetry

Research question: To what extent do adult tope use the waters within and outside of the Welsh Zone during different seasons and what environmental variables are associated with this movement?

Null hypothesis: Adult tope remain in shallow waters in the Welsh Zone year-round.

Alternate hypothesis: Adult tope show large variation in movements outside of the Welsh Zone, following a period of reproduction in shallow coastal waters.

N = 30. Discussions with colleagues have concluded that 30 tope would be an optimal number of individuals to tag for the first satellite tagging study for tope in Wales, to capture robust data to inform future research. It also aligns with practical constraints due to the cost of tags and the number we can secure in grant funding applications. However, the use of miniPAT satellite tags maximises the likelihood of gaining high quality and quantity data from each individual, thereby minimising the number needed to gain meaningful results.

Type of study: correlational study

Type of experiment: exploratory study

Type of design: within-subject design

Dependent variable: temperature, pressure, light-levels (inferred location)

Independent variable / factors: sex, length

Randomisation design: Animals are randomly selected through fishing procedure (Source 2).

Blinding design: The person analysing the tag data will be provided coded information for sex, to minimise subjective interpretation of data.

Sex ratio: Our intention is to tag even sex-classes, but given that animals are randomly selected and miniPAT tags have a failure rate of 20-30%; the sex ratio is likely to be different to 1:1. We will tag any tope over 90cm that has been caught and assessed as appropriate to tag through the welfare and vitality assessments, to maximise outputs given the stress caused during the fishing procedure.

Statistical analysis: Analysis will follow that published in Curnick et al. 2020 (detailed above)

Examples of number of animals used in published research: Several published studies on tope satellite telemetry have generated sufficient data using small sample sizes:

Schaber et al. 2022 – 16 tope tagged with pSAT tags

Thorburn et al. 2019 – 1 tope tagged with a pSAT tag

McMillan et al. 2019 – 14 tope tagged with miniPAT tags

Rogers et al. 2017 – 10 tope tagged with pSAT tags

Martin Cuevas et al. 2014 – 5 tope tagged with pSAT tags

Tissue sampling

Research question: To what extent are angelshark using the Welsh Zone connected with other populations in the UK and Europe? What is population structure of Tope utilising the Welsh Zone and to what extent are they connected with other populations in the North East Atlantic and Mediterranean Sea?

Null hypothesis: Angelsharks and tope have no connectivity with other population(s) in the North East Atlantic and Mediterranean Sea

Alternate hypothesis: Angelsharks in Wales and Ireland are closely related, but do not show connectivity with populations in the Mediterranean Sea nor Canary Islands; Tope from Wales are strongly connected with Tope utilising waters across the North East Atlantic and Mediterranean Sea.

N = 78 (18 angelsharks, 60 tope). Tissue samples will be taken for genetic analysis from all animals tagged with miniPAT or acoustic tags; in addition, tissue samples will be taken opportunistically on up to 6 angelsharks encountered via snorkel/scuba diving (Source 3) and up to 30 tope when attending normal charter fishing trips taking paying clients to target tope (Source 4), to make best use of these encounters

Type of study: correlational study

Type of experiment: exploratory study

Type of design: factorial

Dependent variable: genetic markers

Independent variable / factors: sex, length

Randomisation design: Animals are randomly selected if encountered.

Blinding design: The person analysing the genetic data will be provided coded information for sex, to minimise subjective interpretation of data.

Statistical analysis: Microsatellite analysis of tissue samples will be completed following methodologies outlined in Feldheim et al. 2001 and Plank et al. 2021. Specific statistical methods to compare individuals and/or populations will depend on the number and location of tissue samples collected in this project and through collaboration with researchers in other parts of the species range. For such rare species, any genetic

analysis of tissue samples will provide vital information on ecology and connectivity to inform conservation.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We conducted detailed literature review and organised meetings with colleagues working on elasmobranch telemetry for these species to ensure our relatively low sample sizes would still enable robust analysis to ensure the research can meet our objectives. During experimental design, we chose to apply both miniPAT and acoustic tags onto some individuals (3 angelshark) to minimise the total number of animals involved in our study. Further, we have designed our studies to optimise the data quality and quantity obtained from each individual through using well-tested tag manufacturers.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Tissue samples collected for genetic analysis will be combined with samples taken by other research groups working on these species across their range, so that analyses can be conducted at a local-to- global level and make the best use of the samples. In addition, each tissue sample will be sub-sampled and preserved so that they can be shared in the future with other research groups to reduce the need to go and sample more wild elasmobranchs.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Every step of the protocols outlined in this programme of work have been carefully refined over the last 10 years using experience of the principal investigator and co-investigators, knowledge-exchange with project partners, literature review and through running collaborative workshops to assess best options for tag attachment. This has been particularly pertinent for angelshark telemetry, as the principal investigator and co-investigators are part of the first project to complete telemetry on this species (Angel Shark Project). As such, all tagging methods have involved a multi-stage design and refinement process over several years, which has involved compile expertise from various sources. The principal investigator has led this process for visual ID, acoustic and miniPAT tag attachment design. Outlined below are some of the key refinements for each step in the protocols:

Source

Close collaborative working with experienced charter boat fishers identified that hook and line would be more selective and could be fished in a way to target either tope or

angelshark. This was demonstrated in the 2021 angelshark pilot survey, which was successful in encountering 1 angelshark and had 0 bycatch after 7 hours of fishing effort. Charter boat fishers have the most experience in targeting elasmobranchs to minimise the amount of time each animal is “fighting” on the hook.

Circle hooks were chosen (as opposed to the ‘classical’ J-hooks) as they minimise harm to any elasmobranchs caught through reducing the chance of the elasmobranch swallowing the hook and it becoming embedded deep within the throat, where it becomes difficult or impossible to remove without a lot of damage.

Strong lines were chosen to minimise chance of the line snapping and the hook remaining inside the animal.

Handling/Restraint

Wherever possible, tope will be tagged whilst still in the water alongside the boat to minimise stress caused to the animal by bringing it out of the water. However, when sea conditions prevent this from being possible they will be onboarded to tag (angelsharks will always be onboarded as their shape make it impossible to tag alongside the boat) and the following refinements have been used to minimise impact on welfare and biology:

Placed on a foam mat

Damp cloth used to cover eyes

Saltwater continuously pumped over the gills

If a sunny day, a tarpaulin will be set up so that shark is kept in shade.

Unhooking conducted by the charter boat skipper with significant experience

When moving the elasmobranch, its ventral surface will be supported and/or a landing net or sling will be used

In addition, following discussions with colleagues, it was decided that no anaesthesia will be used as it is common practice not to use anaesthetic/analgesic in external attachment of tags to elasmobranchs as any benefit would be outweighed by the increased amount of time the animal is out of the water.
Further, no-one has anaesthetised an angelshark before, therefore there is no information on anaesthetic concentration, amount of time to anaesthetise or amount of time needed to recover after anaesthetic.

Satellite telemetry (Procedure 1)
The full 2021 workshop report for angelshark satellite telemetry is provided in Appendix 3.

<u>Tag choice:</u>

Wildlife Computer MiniPAT tags were chosen for this procedure as a) they are satellite-linked giving greatest likelihood that we will retrieve data from the small number of sharks tagged (rather than relying on data storage tags which would detach and float in the current and if washed onto shore rely on being found by someone); b) they are smaller (124mm length) and lighter (60g weight) compared with other tags with comparable functionality (e.g. PSAT tag “PAT 5” has 330mm length and 75g weight).

Tag location:

The posterior margin of the left pelvic fin was chosen for angelsharks as a) it is away from the body cavity that houses all the internal organs (removing chance of injuring vital organs); b) it is the part of the body that has the least muscular movement (which reduces likelihood of abrasion); c) it preferred over use of the pectoral fins, as these are used in both burying or mating behaviour; d) it is far away from the head to minimise likelihood that the buoyant miniPAT tag will influence ambush predation; e) it has good muscle thickness for attachment of a ST1 sure-tag between the fin rays (see image below).

The dorsal surface of the skin adjacent to the base of the first dorsal fin was chosen for tope as this is where the abdominal wall is the thickest (significantly reducing chance of injuring vital organs), and is a standard approach for tope satellite telemetry (see image below).

Tag attachment:

Several attachment methods were discussed during the 2021 workshop (bespoke steel cable harness, umbrella-head dart tag, metal-head dart tag, Modified rototag, Modified T-bar tag, Plastic-head dart tag, Modified petersen disc), but the ST1 Button sure-tag was chosen as most suitable as they do not require a surgical procedure, are quick to attach, have good retention rate and have been successfully applied on common skate for the same purpose. During trials on angelshark fins (completed during a dissection of an animal found dead, washed up on the beach), we trialled attaching the ST1 Button sure-tag directly, following a small incision by a scalpel and following a small incision by a drill; it was found that using a drill made a much cleaner wound and prevented the tough Angelshark skin pinching around the tag pin (see image below) – this method of incision is often used for common skate (Cefas, pers comms). In addition, it was decided to have two pin sizes (standard pin and long pin) prepared with a miniPAT tag on the boat to choose the appropriate size to ensure fit was suitable for the thickness of the pelvic fin (to reduce chance of squeezing/pinching and/or abrasion).

For tope, a metal-head dart tag attachment was chosen as this is commonly used in scientific research to instrument miniPAT tags onto medium-sized shark species.

Acoustic telemetry (Procedure 2)

Tag placement:

External acoustic tag attachment was chosen for angelsharks as this has demonstrated success in the Canary Islands, where the Angel Shark Project has fitted acoustic tags onto 112 adult angelsharks (Mead et al. in prep; Barker et al. in prep) (See image below). The development of the modified cattle tag and tag applicator was completed over a 24 month period, with three prototype testing periods in December 2017, March 2018 and May 2018, with final product testing in July 2018 and further refinements between November 2018 to December 2019 based on a small number of resightings of

healthy, tagged individuals. Full details of the development, prototype testing and resulting tag attachment and applicator are provided in Appendix 4. The focus of this development period and rigorous testing was to ensure that the resulting tag attachment caused

minimal stress to Angelsharks during application and had the minimal impact on Angelshark ecology after the procedure.

Visual ID tag (non-regulated) - Procedure 3

The T-Bar anchor tags will be inserted at a 45° angle to minimize likelihood of puncturing the body cavity or vertebral column.

Tissue sampling

Taking a tissue sample for genetic analysis from the tip of the second dorsal fin was chosen because it minimises pain, as few nocireceptors are located on the fins.

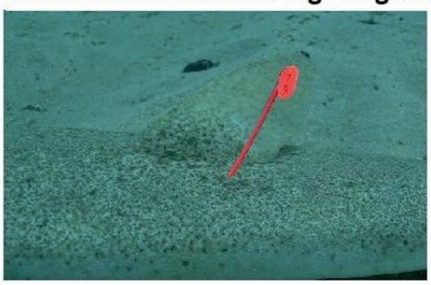
Related images:

Procedure 1: Satellite tag - angelshark

Procedure 1: Satellite tag - tope

Procedure 2: acoustic tag - angelshark

Procedure 3: visual ID tag - angelshark or tope



Why can't you use animals that are less sentient?

The species and lifestages of elasmobranchs chosen are of specific interest in this programme of work so they cannot be replaced.

No Anaesthesia or analgesic will be used following discussions during a workshop held to design the miniPAT tagging methodology ("2021 Workshop"), which concluded that it is not appropriate for the following reasons:

Anaesthetising elasmobranchs during tagging in the field carries a higher risk than working to release the fish as quickly as possible after capture and instrumentation,

No-one has anaesthetised an angelshark before, therefore there is no information on anaesthetic concentration, amount of time to anaesthetise or amount of time needed to recover after anaesthetic. The risk of getting the concentrations wrong and killing the angelshark would be too high.

It is common practice not to use analgesic in external attachment of tags to elasmobranchs as any benefit would be outweighed by the increased amount of time the animal is out of the water.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Welfare and vitality assessment scoring charts (Appendix 1 and image below) have been adapted from standard Cefas protocols, so that they are bespoke to the target elasmobranchs in this programme of work. They will primarily be used at two points in the protocol – the first as soon as the animal is restrained to assess whether the animal is in a suitable condition to start the procedure; the second at the end of the procedure to assess whether the animal is fit for release to the wild

In total, 12 Clinical signs will be included in the welfare assessment and scored between 0 and 3:

- Appearance - Body condition (Nutritional state of the animal)
- Appearance - Skin condition (Bleeding: obvious bleeding from any location.)
- Appearance - Skin condition (Wounding (nicks or shallow cuts on body) or skin lesions (Large cuts or lesions))
- Appearance - Skin condition (Abrasion: Haemorrhaging red area from abrasion.)
- Appearance - Skin condition (Net marks: any type of clear, visible net marks on the body.)
- Appearance - Skin condition (Scar tissue: any scars around the mouth or fin margins which are healed over.)
- Appearance - Discharge (Ocular; nasal; uro-genital)
- Appearance - Parasites (Parasite load)

- Body functions - Respiration (Spiracle pumping frequency)
- Behaviours - Movement (Flapping of fins, movement of body, or movement of tail once placed on the wet mat with eyes covered)
- Behaviours - Movement (Head rearing upwards (Angelshark specific) or attempt to bite (other elasmobranch))
- Procedure-specific indicators - Reaction (Reaction to tag)
- **Vitality will be assessed using three clinical signs scored as either 0 (absent) and 1 (present):**
- Body Flex - Stimulus: shark is laid flat and held at the top of the pectoral fins (angelshark) or behind the head (tope) | Reflex response: active body motion including tail, head or fin movements
- Startle Touch 1 - Stimulus: the top of the pectoral fin (angelshark) or the side of the shark above the lateral line (tope) is ‘swept’ with a smooth blunt object | Reflex response: active body motion including tail, head or fin movements
- Startle Touch 2 (just angelshark) - Stimulus: the side of the tail is ‘swept’ with a smooth blunt object | Reflex response: subtle body tremor or shiver
- Startle Touch 2 (just tope) - Stimulus: the shark is gently tapped on the head between the eyes with a smooth blunt object | Reflex response: subtle “flinch” of the head.

	Measure of welfare	Clinical sign	Description	When assessed	weighted?	Score = 0	Score = 1	Score = 2	Score = 3
WELFARE ASSESSMENT	Appearance	Body condition	Nutritional state of the animal	Once at beginning	Y	Normal body shape	Body shape slightly less-rounded/plump	Body shape looks flat or slightly concave	Body shape is concave; outline of cartilaganous skeleton visible; thin
	Appearance	Skin condition	**Bleeding:** obvious bleeding from any location.	Once at beginning	Y	Absent	Light bleeding at one site	Moderate bleeding at one site or light bleeding at several sites	Heavy bleeding at one site or moderate bleeding at several sites
	Appearance	Skin condition	**Wounding** (nicks or shallow cuts on body) or **skin lesions** (Large cuts or lesions)	Once at beginning	N	Absent	Wounds at one to several sites	Medium lesion at one site or small lesions at several sites; and/or several small to medium sized wounds at several sites.	Large lesion at one site or medium lesions at several sites; and several other medium to small lesions/wounds.
	Appearance	Skin condition	**Abrasion:** Haemorrhaging red area from abrasion.	Once at beginning	N	Absent	Small area of abrasion at one to several sites	Several small to medium sized areas of abrasion at several sites.	Body covered in lesions, some particularly large
	Appearance	Skin condition	**Net marks:** any type of clear, visible net marks on the body.	Once at beginning	N	Absent	light net marks present in small areas	net marks present in small patches across multiple areas	deep net marks present in multiple areas
	Appearance	Skin condition	**Scar tissue:** any scars around the mouth or fin margins which are healed over.	Once at beginning	N	Absent	Small area of scar tissue at one to several sites	Several small to medium sized areas of scar tissue at several sites.	Body covered in scar tissue, some particularly large
	Appearance	Discharge	Ocular; nasal; uro-genital	Once at beginning	N	Absent	Small amounts of discharge at one location	Small to medium amounts of discharge from several locations	large amounts of discharge from several locations
	Appearance	Parasites	Parasite load	Once at beginning	N	Absent	1-5 parasites present	6-20 parasites present	>21 parasites present
	Body functions	Respiration	Spiracle pumping frequency	Throughout duration on boat	N	spiracles pumping normally	Spiracles pumping slower or faster than normal	spiracles pumping much slower/faster than normal	spiracles pumping erraticaly or locked open
	Behaviours	Movement	Flapping of fins, movement of body, or movement of tail once placed on the wet mat with eyes covered	Throughout duration on boat	N	normal reactive movement response to stimulus	occasional short-duration movements without stimulus	regular large or erratic movements without stimulus (repeated short; or long duration)	constant erratic movemenhts OR no movement to any stimulus
	Behaviours	Movement	Head rearing upwards (**Angelshark specific**) or attempt to bite (**other elasmobranch**)	Throughout duration on boat	N	Absent	occasional head-rearing / bite attempt	frequent head-rearing / bite attempts	regular, repeated head-rearing / bite attempts
	Procedure-specific indicators	Reaction	Reaction to tag	During tag application	N	Absent	small reactive movement after tag attachment	medium reactive movement after tag attachment	large and prolonged movements after tag attachment
	Other relevant observations	**Notes:**							

Total score	Action to take
0 to 2	Normal (no abnormalities, a score of 1 in two signs is unlikely to be of significance). Start procedure as planned.
2 to 10	Mild signs, monitor more closely. Start procedure as planned.
11 to 27	Easily recognisable signs of poor health and/or welfare, monitor more closely. Decision whether to start the procedure depends on the specific scores and overall condition of the shark. • If assessed in a suitable condition/welfare, start procedure as planned but with closer monitoring. • If assessed as NOT in a suitable condition/welfare to start procedure, return to the wild without the procedure or complete humane endpoint as outlined below.
28 to 35	Marked signs of poor health and welfare, do not start the procedure. Decision whether to release to the wild or complete humane endpoint depends on the specific scores and overall condition of the shark. • If assessed that the humane enpoint is the right approach, completed Schedule 1 killing. • If assessed that the shark has some chance for survival, release back to the wild before tagging.
36	Hypothetical maximum score. Utilise humane endpoint through Schedule 1 killing.

T	Measure of welfare	Clinical sign	Description of stimulus	Reflex response	0 (absent)	1 (present)
VITALITY ASSESSMENT	Vitality BEFORE procedure	Body Flex	Stimulus: shark is laid flat and held at the top of the pectoral fins	active body motion including tail, head or fin movements		
	Vitality BEFORE procedure	Startle Touch 1	ANGELSHARK ONLY Stimulus: the top of the pectoral fin is 'swept' with a smooth blunt object TOPE ONLY Stimulus shark is gently tapped on the head between the eyes with a smooth blunt object	ANGELSHARK: subtle body tremor or shiver TOPE: subtle 'flinch' of the head		
	Vitality BEFORE procedure	Startle Touch 2	Stimulus: the side of the tail is 'swept' with a smooth blunt object	subtle body tremor or shiver		
				TOTAL VITALITY SCORE BEFORE PROCEDURE		
	Vitality AFTER procedure	Body Flex	Stimulus: shark is laid flat and held at the top of the pectoral fins	active body motion including tail, head or fin movements		
	Vitality AFTER procedure	Startle Touch 1	ANGELSHARK ONLY Stimulus: the top of the pectoral fin is 'swept' with a smooth blunt object TOPE ONLY Stimulus shark is gently tapped on the head between the eyes with a smooth blunt object	ANGELSHARK: subtle body tremor or shiver TOPE: subtle 'flinch' of the head		
	Vitality AFTER procedure	Startle Touch 2	Stimulus: the side of the tail is 'swept' with a smooth blunt object	subtle body tremor or shiver		
				TOTAL VITALITY SCORE AFTER PROCEDURE		
	Other relevant observations	**Notes:**				

Total score	Action to take
0	**Dead** Before procedure: Ask authorities for scientific dispensation to land shark and complete dissection at a later date to gather data. After procedure: Ask authorities for scientific dispensation to land shark and complete dissection at a later date to gather data.
1	**Poor condition** Before procedure: Decision to a) start the procedure, b) return to the wild without the procedure or c) complete humane endpoint depends on the Welfare Assessment and overall condition of the shark. After procedure: Decision to a) return to the wild or b) complete humane endpoint depends on Welfare Assessment and overall condition of the shark.
2	**Good condition** Before procedure: Start procedure, but with close monitoring. After procedure: Return to the wild.
3	**Excellent condition** Before procedure: Start procedure as planned. After procedure: Return to the wild.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The procedures outlined in this programme of work follow best practices developed by Cefas, but we also keep up to date with animal welfare literature and practices. All activities involving animal use at ZSL are reviewed by an ethical review committee.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The principal investigator and co-investigators are part of a global network of researchers who conduct similar research and we regularly exchange ideas and learn from each other's approaches. This will be augmented as part of this programme of work by organising a focal meeting on animal welfare with the Project SIARC Research Group. The project team will also reach out to researchers working on the same species in other areas to discuss and share refinements used to improve elasmobranch welfare during the tagging process.

49. Breeding of genetically altered rodent strains

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Genetically altered, Breeding, Immunodeficient

Animal types	Life stages
Mice	juvenile, adult
Rats	adult, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The purpose of this license is to breed and supply genetically altered rodents and ova/embryos as a service to research establishments that lack the expertise, capacity or facilities in their own establishment to conduct this work and to supply to other projects that have the authority to use these types of animals.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The provision of these services under this licence will allow important programs of scientific research to progress more rapidly, and thus increase the speed at which new potential therapies for human and animal disease can be identified and developed.

Through provision of centralised breeding facilities it is possible to ensure that the genetically altered rodents are maintained in a high health status environment using best husbandry practices under the care of well trained personnel. This will minimise suffering and the likelihood of adverse or confounding effects of biological contaminants on the animals. Standardisation helps to reduce experimental variability, increasing the significance of results and reducing the number of animals needed in a program.

A centralised breeding facility meeting the needs of several programs also makes it possible to finely manage the colonies to ensure that only the optimal numbers of animals are produced to support the researcher's studies.

What outputs do you think you will see at the end of this project?

Ensuring that the genetically altered rodents are produced in a timely, reproducible manner, with a high health status by expert staff will minimise any suffering and the likelihood of adverse effects, reducing the numbers of animals produced.

Generation of embryos/ova and supporting rederivation will allow for the generation and maintenance of rodent strains at higher health status removing confounding effects of these organisms and any clinical implications for animal welfare.

Provision of superovulated plugged mice or embryos for Mouse Embryo Assay (MEA) from centralised colonies allows significant time saving for customers, reproducibility of the model and reduction of the numbers of mice used.

Through the provision of centralised colonies and supporting cryopreservation the total numbers of genetically altered animals produced will be reduced.

Achievements in terms of scientific outcomes remains with our clients - the end users of the rodent or rodent products.

Who or what will benefit from these outputs, and how?

This provision of these services under this licence will allow important programs of scientific research to progress more rapidly, and thus increase the speed at which new potential therapies for human and animal disease can be identified and developed.

How will you look to maximise the outputs of this work?

We will work closely with the client in ensuring all data and information on the breeding and supply of the rodents meet the scientific needs of the research. Any learning from the breeding of the genetically altered line will be shared with the client for use in their scientific arena and aid collaboration of new knowledge.

We fulfill these requirements in support of the scientific community, as a commercial service providing efficient breeding strategies. We have invested and continue to invest considerably in both dedicated facilities and specific training in the welfare and management of genetically altered rodents.

Species and numbers of animals expected to be used

- Mice: 1,115,000

- Rats: 20,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will be using juvenile and adult rodents (mice and rats) under this licence, of which >95% will be genetically altered.

They are the most refined models for the intended purpose due to the ability to experimentally alter the genome. Genes can be injected directly into the fertilized egg of a rodent creating what is known as a transgenic animal. This approach allows scientists to create new models and experimental tools based on the manipulation of specific genes thought to be important in the pathology of certain diseases.

Scientists have developed techniques that allow them to specifically target genes within the rodent genome –so-called “knockouts” – that have further enhanced their biological toolkit.

Almost all human genes known to be associated with diseases have counterparts in the rodent genome and appear highly conserved through mammalian evolution, making them excellent models for many areas of medical research.

Typically, what will be done to an animal used in your project?

Breed and supply a range of genetically altered rodents to bona fide scientific research establishments in order to support multiple scientific objectives. This would include generating animals, developing models through intercrossing/backcrossing and appropriate health management of the animal colonies.

Provide superovulated or superovulated and mated rodents, early stage embryos, or ova from a range of different genetically normal, harmful mutant and genetically altered rodents to bona fide scientific research establishments. Animals will experience mild, transient pain and no lasting harm from administration of hormones by injection (intraperitoneal).

Maintain, observe, genotype, phenotype and analyse a range of genetically altered rodents on behalf of bona fide scientific research establishments. This would include observing basic phenotype, tissue and body fluid sampling and appropriate health management of the animal colonies. Identification, ear tissue and blood sampling methods may experience mild, transient pain and no lasting harm.

Animals may undergo non-recovery anaesthesia for the purposes of terminal sampling of tissue or perfusion of a tissue fixative, these animals will 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?

Many of the animals will develop no more than mild clinical signs during their lifetime. Others may have the potential to develop more severe phenotypes but will not be kept for long enough for this to happen. The majority of animals will be bred, maintained and, if necessary characterised, under a protocol with a mild severity limit.

A small proportion of GA models will have an adverse phenotype as a model for the area of research, for example cancer, diabetes, and liver disease. These models will be maintained to a moderate severity. Some of these models may develop tumours, have pathological abnormalities, e.g. congential eye abnormalities, have abnormal gait, and/or experience weight loss of up to 15% of bodyweight compared to the normal growth curve for the strain.

Any animals that develop tumours will be monitored closely to ensure the tumour does not impede normal behavior and do not exceed 10mm in diameter.

Animals with a genetic predisposition to/or genetically altered for diabetic disease models will be closely monitored to ensure bedding is changed frequently due to increased urination, and/or blood glucose levels may be measured by pricking a superficial vessel to track the progress of the disease. Animals will normally be shipped to the client before they are expected to exceed moderate severity.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Breeding of GA Mice 60% sub-threshold, ~40% mild, <1% moderate. Breeding of GA Rats 60% sub-threshold, 40% mild.
Superovulation of Mice and Rats 100% mild.

What will happen to animals at the end of this project?

- Killed
- Used in other projects
- Rehomed
- 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?

Animals are essential for research into human and animal diseases as behavioural models and full system responses cannot be replicated using in vitro methods.

Genetically altered rodents allow mechanisms of artificial control of a gene or gene elements and the potential for introduction of foreign genetic material. Clients use these methods to study gene activity in a complex physiological environment in order to

understand the function of particular genes and the action of potential therapeutics in the development or prevention of disease.

Which non-animal alternatives did you consider for use in this project?

It is not possible to use non-animal alternatives to generate GA rodent models for research. However, clients will give consideration and provide justification to the use of non-sentient alternatives, and if unable to replace the use of live animals, we/they will work to identify the most appropriate reduction strategies for the research work.

Why were they not suitable?

Animals are essential for research into human and animal diseases as behavioural models and full system responses cannot be replicated using in vitro methods.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers of animals proposed for use in this project are taken form the numbers used in the previously approved project licence.

The outsourcing of breeding GA rodents into a centralised facility has continued to grow as a cost efficient and standardised approach to ensure expertise and health security is optimised within GA colonies. It is anticipated that outsourcing will continue to grow to minimise the economic burden on maintaining inhouse colonies.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Accurate, standard and concise collection of rodent information and program requirements with data will be sought from clients, suppliers and standard databases (for example the MGI website). This allows the experienced Production/Research Services Departments to make accurate forecasts and production plans which will optimise numbers of animals, ova or embryos generated.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

A benefit of using a centralised service is that animal numbers can be well-controlled and the production of surplus is minimised. A centralised service reduces the potential for researchers to hold multiple in-house colonies across their facilities and thereby reduces the overall number of animals required to produce genetically altered models.
We are additionally able to advise on best breeding regimes, and as we offer services, such as cryo- preservation (freezing of sperm, or embryos) to maintain lines instead of holding multiple 'tick-over' maintenance colonies.

Wastage is reduced through careful management of colonies using bespoke software inventory and tracking systems and through careful forecasting of requirements with customers.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 utilising genetically altered rodents (mice and rats) under this licence. They are the most refined models for the intended purpose due to the ability to experimentally alter the genome.

The majority of animals used under this licence will not experience any pain, suffering or lasting harm due to the way the colonies are maintained within biosecure environments.

Why can’t you use animals that are less sentient?

Genes can be injected directly into the fertilized egg of a mouse creating what is known as a transgenic animal. This approach allows scientists to create new models and experimental tools based on the manipulation of specific genes thought to be important in the pathology of certain diseases.

Scientists have developed techniques that allowed them to specifically target genes within the mouse genome – so-called “knockouts” – that further enhanced their biological toolkit. Modern techniques have developed to allow the genetic alteration of rats which provides further refinement for particular study requirements. For example, almost all human genes known to be associated with diseases have counterparts in the rat genome and appear highly conserved through mammalian evolution, confirming that the rat is an excellent model for many areas of medical research. Behaviourally, rats have a higher level ability to learn and accomplish different experimental tasks. Because of their larger size it is also much easier to perform surgical procedures and monitor physiological states in rats than in mice.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals will be housed in compliance with the Home Office Animal Scientific Procedures Act Code of Practice (CoP) legislation. As the CoP does not currently specifically call out housing floor space requirements for mating genetically altered (GA) animals, we will usually house GA strains with a regular litter size of 8 or less as breeding trios (1 male paired with 2 females) in a compliant breeding cage as for non-GA inbred strains. Strains with a higher regular litter size (similar to what might be seen in a non-GA outbred strain) will not be housed as breeding trios, except in larger cages with a floor area compliant with the Code of Practice.

Animals are kept in biosecure enclosures and sterilised diet, bedding and water is used, this ensures a high health status is maintained. We have a policy that wherever possible, non aversive handling techniques are utilised when handling animals, such as cupping or scooping.

Complete records of the health screening, welfare observations and productivity of the rodent lines will be maintained. The quality will be assessed via the ability to deliver animals, embryos or ova appropriate to the study or process requirements.

Rodents will be observed appropriately to ensure they are maintained to humane endpoints as detailed in this licence. All colonies are maintained within a proprietary software solution for inventory and colony management. This software package tracks all animals from birth to fate as well as clearly detailing any welfare and phenotype indicators the people working on the animals need to be aware of.

Working with customers and colleagues within our organisation, we will continually assess the appropriateness of the techniques and equipment utilised for these protocols.

We are aligned with the Genetically Altered Animals (GAA) assessment framework, which details methods to review, implement and assess the best possible compliance with the 3R's when breeding and using GAA's.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow guidance from the client on their model requirements, however we will continue to regularly check information on the NC3R's website and are signed up to the NC3R's newsletter. We review publications on the laboratory animals science association (LASA) website, as well as attending and contributing to seminars and meetings with LASA.

We are aligned with the Genetically Altered Animals (GAA) assessment framework published by the Home Office.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our staff undergo their own continual professional development programmes and keep abreast of new developments which may facilitate refinement by attendance at meetings and conferences and by following the activities of organisations such as the NC3Rs.

All clients are required to justify their projects scientifically for review and approval by our animal welfare body prior to accepting any work at our facility.

50. Mechanisms and modulation of chronic inflammatory responses in kidney disease- associated pathologies

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Inflammation, Chronic Kidney Disease, Atherosclerosis, Alzheimer's Disease, Infections

Animal types	Life stages
Mice	adult, juvenile, embryo, neonate, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to better define the chronic inflammatory mechanisms that promote the development of major comorbidities in patients with Chronic Kidney Disease (CKD) and to evaluate potential therapeutic approaches to target these. Comorbidities studied here will be atherosclerosis (the main cause of cardiovascular events), Alzheimer’s Disease (AD), and secondary immunosuppression, all of which CKD patients are at increased risk of developing.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Chronic kidney disease (CKD) affects up to 15% of the global population and its prevalence is growing. It can progress to end-stage renal disease, which requires dialysis

or a kidney transplant. In addition, CKD makes patients more at risk of developing other conditions, notably:

- Cardiovascular disease (CVD): At all stages, CKD is associated with markedly increased CV morbidity and mortality. Over half the patients with end-stage renal disease will die of CVD. Notably, atherosclerosis, an inflammation-driven thickening of the vascular wall which underlies most CV events, is aggravated in CKD. Therapies that effectively reduce atherosclerosis in the general population are mostly inefficient in patients with CKD.

- Alzheimer's Disease: 10 % of over-65s have developed AD, and individuals at all CKD stages are at higher risk. AD dramatically reduce life expectancy and quality of life, even more so in CKD patients who may become unable to properly care for their kidneys, take their medication or carry out dialysis as a result of progressing dementia. There is currently no cure for AD and treatments mostly reduce symptoms.

- Secondary immunosuppression: Infections are a major cause of death in advanced CKD (2nd after CVD). Decreased immunity is a major risk factor for infection in CKD, and, as resistance to antibiotics is growing, complementary strategies to help resolving infections efficiently are needed.

Co-morbidities in CKD are associated with a substantial financial healthcare burden. NHS England spends £1.5 billion/year to cover CKD-associated treatments, including CVD and infections, and over
£5 billion/year for dementia.

Recent clinical guidelines highlight the need to move away from single disease management towards an inclusive approach of clustered pathologies to better tackle the multimorbidity issue. To achieve this, a better understanding of the causal relationships between clustered morbidities is required

What outputs do you think you will see at the end of this project?

This project will provide:

1- Novel information about the inflammatory/immune mechanisms that promote the development of 3 major co-morbidities in CKD: i) Atherosclerosis, ii) Alzheimer's Disease, iii) Secondary Immunosuppression.

2- The evaluation of at least one potential therapeutic strategy against the development of the CKD co-morbidities listed above. More strategies may be evaluated depending on the findings made in 1.

3- The dissemination of the above findings in peer-reviewed journals and/or at conferences.

Who or what will benefit from these outputs, and how?

Short term/within project period:

Academic and clinical researchers in the field will benefit from:

- the improved knowledge as a result of this work in the short term – or within the duration of the proposed project.

- the development and characterisation of in vivo models combining CKD and atherosclerosis or CKD and AD. These models may be reproduced by others, under their own licences and in their own establishments. We will also consider running the models under the present licence as part of collaborations when the work fits the aim and objectives of the present licence.

Long term:

Patients and clinicians: The ultimate aim of this project is to enable the development of novel therapeutic strategies against CVD (atherosclerosis in particular), AD and secondary immunosuppression in patients with CKD. In addition, this research will evaluate whether the same therapeutic strategy could be used to reduce both atherosclerosis and AD in CKD.

Thus, this project will facilitate the development of treatments that target multiple, rather than single, conditions. This is in line with recent clinical guidelines towards more inclusive approaches of clustered pathologies, therefore reducing treatment burden and improving outcomes especially for multimorbid CKD patients.

How will you look to maximise the outputs of this work?

We will maximise the outputs of the animal work under this licence by several approaches that are already in place in within our team:

- Results will be discussed at least 4 times/ year within the wider research group, as part of weekly lab meetings.

- Results will be reported in ARRIVE compliant journals. We will aim for high-impact international journals

- We will aim to present the results once yearly at international conferences

- We will aim to report the setting-up of combined atherosclerosis in CKD or AD in CKD in vivo models at the time that it is achieved, prior to generation of new knowledge using these models. These publications will include details of unsuccesful approaches, including inadequate nephrotoxin regimen/ sub-optimal experiment duration or time points/ excessive severity.

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.

Adult mice will be used in our experiments. The work under this licence aims to help the development of therapies that improve outcomes for kidney disease patients, notably by reducing their abnormally elevated risk of cardiovascular disease and dementia or by helping their impaired immune system to fight infections. Our focus is on the role of the immune system in driving this risk. The immune system of humans is much closer to that of other mammals than non-mammals and non-adult mammals tend to not have a fully mature immune system. Therefore, to improve the relevance of our in vivo findings to the human setting, adult mammals need to be used.

Because mice do not naturally develop cardiovascular disease or dementia over their lifespan, we will be using strains of mice that have been genetically modified to promote the development of these diseases.

Typically, what will be done to an animal used in your project?

To reproduce chronic kidney disease in mice, we will do 4 injections of a nephrotoxic substance, into the peritoneal cavity, with a very small needle. Mice will then typically be kept for a duration between 1 and 3 months, more exceptionally 6 months. During that time, they may:

- have a small cut make to their tail to draw small volumes of blood, not more than twice a month.

- be injected with the therapeutic compounds that are being tested, the number of injections will vary, but will be no more than twice weekly.

Depending on whether we are looking at the effect of kidney disease on cardiovascular disease (1), dementia (2), or response to infections (3), mice may also experience ONE of the 3 scenarios below:

- be fed a diet that contains elevated amounts of fat, to raise their blood cholesterol levels and reproduce the development of atherosclerotic plaque seen in humans

- be kept in individual cages for a small period of time, so their capacity to make organised nests can be evaluated. This is a typical measure of cognitive impairment in mice with Alzheimer's disease.

- be given an injection of bacteria, to study how their immune system responds to the infection

What are the expected impacts and/or adverse effects for the animals during your project?

The adverse effects experienced by an animal will depend on the pathology/combinations of pathologies that will be induced:
1- Chronic nephropathy: The only visible clinical sign is a temporary weight loss following the repeated administration of nephrotoxin. Most animals will lose about 10% of their initial weight, while a small proportion may lose up to 15%. Weight loss is temporary (~ 10 days) and is quickly followed by a regain and normal weight gain thereafter.

2- Alzheimer's disease: These animals do not display any clinical sign in the first 6 months of life, during which most of our experiments will take place. Cognitive impairment becomes measurable in special tests after 6 months, but do not affect the day-to-day

welfare of the animals. The tests used to measure loss of cognitive functions will be non-aversive (evaluation of nest structure or sweetened-water preference), and will not induce adverse effects.

3- Atherosclerosis: When prone mice are fed a diet high in fat, they will start developing atherosclerotic plaques, similar to those observed in humans. Atheroslerosis will be silent in >98% of animals, with no adverse effects experienced. About 1% of animals may experience sudden cardiac death, in line with the pathology observed in humans.

4- Infection: Mice infected with bacteria or bacterial components will experience adverse effects in line with the development of a fever. They will become less active, may feel cold, stop grooming themselves and are likely to eat/drink less. These symptoms are temporary and are expected to ameliorate within 1 day and fully resolve within 2 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)?

1- Chronic nephropathy alone: 85% mild, 15% moderate, due to weight loss

2- Chronic nephropathy + Alzheimer's disease: 85% mild, 15% moderate, as for Chronic nephropathy alone, because Alzheimer's disease is mostly unsubstantial.

3- Atherosclerosis alone: >98% mild, ~ 1% sudden death

4- Chronic nephropathy + Atherosclerosis: 84% mild, 14% moderate, < 2% sudden death

4- Infection: 50% Mild, 50% Moderate

5- Chronic nephropathy + Infection: 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?

Chronic inflammation is a dynamic process involving a number of immune and non-immune cell types and a complex combination of pro- and anti-inflammatory mediators, which is impossible to fully reconstitute in vitro. In particular, the mechanisms by which chronic inflammation links damage to the kidney to damage to other organs, such as the blood vessels, the heart or the brain, is expected to involve resident cells and mediators in the specific organs, as well as the exchange of cells and mediators with the circulatory system. Thus, the chain of events leading from local kidney damage to systemic

inflammation and the damage to other organs cannot be mimicked by non in vivo systems, even very complex ones.

Which non-animal alternatives did you consider for use in this project?

Much of the experimental hypotheses presented here have been driven by data obtained in vitro from human cell culture experiments or by observations made ex vivo in samples from CKD patients.

Each step of the proposed work also encompasses ex vivo and in vitro experiments, either to replace (when possible), help design or complement the proposed in vivo experiments.

Why were they not suitable?

Our proposed ex vivo or in vitro alternatives are suitable for a given specific purpose. However, the use of a dynamic in vivo model remains necessary to address the precise role of specific immune pathways in the clustering of multi-morbidities in CKD and to investigate the therapeutic potential of targeting these pathways.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Experimental animals: In experiments under the previous licence that used the same core model of chronic nephropathy, we have observed that a group size between 3 and 6 animals is necessary to obtain robust and reliable results. This variability in the required group size is linked to the readout of interest. For example, we have observed that kidney fibrosis is strongly elevated in that model and may only require 3 animals/ group to measure a statistically significant difference with control, while plasma creatinine levels may be less strongly elevated or more heterogenous, requiring a group size of at least 5 to reach statistical significance. Many of the readouts to be tested here, notably those linked to atherosclerotic plaque measurements or Alzheimer's Disease quantification, have not been tested in a combination with chronic nephropathy before. Therefore, and unless our pre-existing data suggests that a lower group size is sufficient, estimations for unknown readouts were based on a group size of 6.

Estimates also account for the fact that some experiments, notably pilot ones, may be repeated.

Breeding animals: Estimates were made under the assumption that all strains used will be good, but not exceptional breeders (5-7 pups/litter), based on the experience of other groups maintaining these same strains, and that each strain will need to be actively bred for a duration of 3 years. All animals bred will have the genetic alteration of interest and both sexes will be used, limiting the number of animals bred.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Whenever possible, experiments requiring the same control groups will be performed at the same time to avoid repetition of the group. If that is not possible, control organs or tissues may be kept frozen for analysis at the time of a later experiment, again with the aim of avoiding control group repetition.

When evaluating a treatment option, we will not run a treatment only (no disease) group for the evaluation of non-specific effects until the treatment has been found efficient in the disease group. This will avoid in depth testing of non-optimal therapeutic strategies.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Small size pilot experiments will be run in order to set up our combined models of chronic nephropathy + atherosclerosis or chronic nephropathy + Alzheimer's Disease, with only two groups (chronic nephropathy vs no nephropathy) of n=3/4 animals/group. Although this likely may be too small to achieve statistical significance, we expect it will be sufficient to determine whether chronic nephropathy has promoted atherosclerosis or Alzheimer's. All readouts of potential interest will be measured in these pilot experiments, so they can be included in the analysis of the full scale follow-up experiments. If none of the pilot animals with chronic nephropathy shows more atherosclerosis or

Alzheimer's disease burden than the non-nephrotoxic control, then the dose of nephrotoxin will be adjusted, as described in the Action Plan.

When it will not invalidate the interpretation of the findings, normal tissues will be obtained from spare animals procured from other groups and identified via the 3R’s Blackboard tick@lab sharing platform. This may include healthy kidneys, aortas or brains for histological comparison with the diseased organs.

Because some of the post-cull techniques will be novel to our group (brain dissection, brain immunohistochemistry), we will become fully familiar with the techniques using spare animals/organs obtained from other groups, as described above. We will not use pilot animals for this.

Potential therapeutic strategies will first be tested for their efficacy human cells or tissues (blood)in blood cells from patients with CKD, the most relevant and most potent target(s) will be selected for in vivo testing and targeting

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

- Nephrotoxin injection to induce chronic nephropathy: Mice will experience transient stress and discomfort at the time of intraperitoneal injection. To reduce that, small insulin needles will be used and repeated injections to the same site in a short period of time will be avoided.

Apart of this, mice do not display signs of adverse effects, other than a modest temporary weight loss. Although the kidneys appear damaged by histological examination, kidney disease is asymptomatic in this model.

- High fat diet for atherosclerosis development: A genetic alteration coupled with a diet is necessary for mice to develop atherosclerosis. The disease is silent is > 98% of the cases. Sudden cardiac death, due to obesity and plaque development, is a rare occurrence but may happen in ~1% of animals. This cannot be avoided given the need to reproduce atherosclerosis progression as it is seen in humans.

- Alzheimer's Disease (AD) model: Normal mice do not spontaneously develop AD over their lifespan. The mice we will use are genetically modified to show histological signs of the disease from 2 months of age, but cognitive functions do not deteriorate until 6 months of age. Loss of cognitive function will be detected by assessing the ability of the animals to make a nest from given materials.
For this, mice will need to be housed on their own for a short period of time (2-3 days). Cognitive decline does not otherwise impact the day-to-day welfare of the animals.

Other models of AD using non genetically modified animals exist, but they require administration of AD- promoting agents or chronic exposure to stress, and therefore have a worse impact on animal welfare.

- Infection with live bacteria: Adverse effects include reduced natural and provoked activity and a lack or grooming for 24h after the injection, which will resolve fully by 48h. We have established optimal doses for several bacterial strains and the minimal dose required to obtain robust results will be used. Reducing the bacterial dose further would reduce the adverse effects but does not lead to a sufficiently immune response to generate reliable findings

Whenever possible, bacterial compounds or heat-killed bacteria, which lead to lower adverse effects than live bacteria, will be used. However, one of our aim is to investigate the effect of chronic nephropathy on bacterial clearance, which will require the use of live bacteria. This is because live bacteria will disseminate from the injection site (peritoneum) into the bloodstream and better reproduces what is seen in patients.

Why can't you use animals that are less sentient?

The ultimate aim of the work under this licence is to help the development of therapeutic strategies that improve outcomes for kidney disease patients. Our focus is on the modulation of immune responses.

The immune system of humans is much closer to that of other mammals than non-mammals and non- adult mammals tend to not have a fully mature immune system. Therefore, to improve the relevance of our in vivo findings to the human setting, adult mammals need to be used.

The immune responses that we are investigating are expected to take weeks (to months) to develop, therefore excluding the use of terminal anaesthesia.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

As indicated above, we have selected as our first choice the most refined and less severe models, for equivalent clinical relevance. That includes the use of i) mild nephrotoxin over more severe ones or over surgical kidney damage; ii) LDLR-/- over ApoE-/- mice, as they have less aggresive atherosclerosis, and iii) AppNL-G-F over other AD strains, as experimental time is reduced for the same severity.

While all animals will be routinely monitored, monitoring will be increased whenever: i) new combinations of protocols are used; ii) new genetic backgrouns are used, iii) new compounds or new doses are used; and iv) live bacteria are administered. Monitoring frequency and duration will be according to the specific risks of the protocols, strains lines or compounds used. For injection of live bacteria, monitoring will be at 4h post-injection and subsequently at least daily until all adverse effects have ceased.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The Animals in Science Regulation Unit (ASRU) Guidance and Regulatory Advice will be referred to for the most up-to-date guidance on refinement. When required, additional advice will be obtained from

the NC3Rs resource library (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?

The home establishment provides regular email updates on 3Rs matters and new initiatives.

51. Nanotechnologies for cancer treatment, diagnosis and monitoring

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

Nanotechnology, Nanomaterials, Therapy, Cancer, Glioblastoma

Animal types	Life stages
Mice	embryo, neonate, pregnant, 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?

Nanomaterials are very small materials with at least one dimension (eg. width, length, height) that is typically less than 100 nanometres (one billionth of a metre) in size. The overall goal of this project is to design and test novel therapeutic, diagnostic and monitoring tools based on nanomaterials for applications 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?

Cancer remains an unmet clinical need that requires new approaches. The use of novel technologies based on nanomaterials (nanotechnologies) seeks to design smarter solutions with potential to overcome several key clinical problems. This includes delivery of therapeutic agents in more targeted and safer ways, use as medical imaging probes to aid clinical diagnosis and monitoring, and tools to identify biomarkers for early detection.

What outputs do you think you will see at the end of this project?

This project will generate new information on the application of nanotechnology in cancer. The new information gained from this project will be shared in the form of scientific publications, conference communications and through public engagement activities throughout this project.

While the timescale for these nanotechnologies to move from preclinical investigation to clinical products are more likely to be in the long term, we aim to build up rationale and justification within this project for clinical testing and validation of these technologies.

Who or what will benefit from these outputs, and how?

These communications will enable collaboration and stimulate further research into advanced biomedical nanotechnologies throughout the scientific community.

Eventually, patients will benefit from any effective new technologies for the diagnosis, monitoring or treatment of cancer that are developed based on the results obtained during this project.

How will you look to maximise the outputs of this work?

Publication and communication of our findings will always be the primary aim of this work. The goal of every experiment conducted under this licence will be to generate valid, high quality and therefore publishable results and we will endeavour to ensure that all findings meeting these criteria will be published (primarily in open access journals) to inform the wider scientific community, even if these do not support the therapeutic, diagnostic or monitoring potential of the particular nanomaterial, or nanomaterial device being investigated.

Species and numbers of animals expected to be used

- Mice: 5100
- 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.

Mice are chosen for many of these studies for a variety of reasons, primarily as they are the least sentient mammalian species that will provide data applicable to humans. The tumour models using this species are already well characterised and validated with close similarities to the human tumours such that the findings and nanotechnologies developed have the best chance of clinical translation and population benefit. Rats are used as an alternative rodent model for some of this work where either the tumour models require this (eg. when using cancer cells of rat origin) or where the larger size of these rodents is beneficial for the nanotechnologies being developed (eg. as imaging agents).

The majority of research will use adult animals as the cancers being investigated are those which primarily affect adults.

Typically, what will be done to an animal used in your project?

For testing of nanomaterials in animals with cancer, tumours will be induced in these animals at an appropriate site for the relevant cancer (eg. under the skin for melanoma, in the brain for glioblastoma, in the lungs or other organs when modelling metastasis). For animals with brain tumours, an additional surgery may be performed to resect (surgically remove) the tumour, to mimic how these tumours are treated in patients.

To measure tumour growth in these animals either direct measurements with callipers (superficial tumours), or live imaging approaches (non-superficial tumours) may be used.

Either before, or during the course of tumour growth, animals will be administered with nanomaterials or a nanomaterial based therapy (either single dose or repeat administrations) or controls via appropriate route(s). The effects of the nanomaterials will be assessed by monitoring tumour growth using the above described methods in combination with other approaches such as imaging, taking

blood samples or collecting urine/faeces samples. We may also combine these nanomaterial therapies with existing therapies such as immunotherapy or tumour treating fields in order to design and develop strategies that could work synergistically with these approaches.

At the end of the experiment, usually before tumour growth is associated with any adverse clinical signs, animals will be humanely killed and tissues and tumours collected for further analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

From our previous work, we don’t expect the nanomaterials we use to have any particularly strong adverse effects. The majority of nanomaterials, or nanomaterial-based therapeutics will have been tested to confirm their biocompatibility in a companion project licence. The procedures used to administer the nanomaterials are usually the least invasive possible. Where surgical administration is needed, this will be done under anaesthesia and analgesia/additional support is provided to minimise the adverse effects associated with this.

Each of the tumour models used may have associated adverse effects. Superficial tumours are placed on sites (eg flank or back) that usually have minimal impact on the animals. For superficial tumours, skin ulceration may occur due to the rapid growth of the tumour or after nanomaterial treatment. These animals will be monitored closely and treated to avoid these ulcers progressing and causing suffering. If these ulcers start to worsen or bleed, animals will be humanely killed.

For metastatic tumours, the tumours may interfere with certain organ functions which is usually associated with weight loss which is used as a humane endpoint. Reparatory distress can occur, for example following the initial injection of cancer cells, but if this does not improve quickly (within minutes) animals would be humanely killed.

For tumours in the brain, animals usually behave normally but may lose weight or show subdued behaviour at the later stages of tumour growth. This is used as a humane endpoint to prevent any prolonged 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)?

Mice: 100% moderate

Rats: 100% moderate

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Before materials or devices are tested in animals, they will first be tested in cell cultures, or in relevant in vitro biological systems to check the safety and to help determine the best doses that may be effective in vivo. Many responses to nanomaterials, including the pharmacology/biodistribution, are driven by complex interactions with multiple cell types as part of whole systems (eg. the immune system, the cardiovascular system etc). These cannot be effectively modelled and integrated in an in vitro setting and therefore require animal testing.

Due to the complex nature of the diseases we are investigating, it would not be possible to test the nanotechnologies we have developed without testing in animals. Cancer is a highly complex disease whereby uncontrolled growth of different populations of cancer cells interact closely with the immune system, generate new vasculature, remodel of the surrounding tissue/organ microenvironment, all of which need to be effectively modelled simultaneously to be representative of the patient populations. When considering the use of nanomaterials as therapeutics, targeting the tumour following administration in the blood stream can only be confirmed in animals with a complete circulatory system.

Which non-animal alternatives did you consider for use in this project?

In vitro cell cultures including 3D models and co-culture systems (up to and including organoids which are 3D in vitro models that contain multiple cell types and mimic organs more closely).

Why were they not suitable?

Non-animal alternatives such as those listed above can provide important information and are always used in the first instance for all new technologies and nanomaterials. This

includes testing in organoid systems which is an ongoing effort by our group. However, none of these systems (including organoids) can effectively recapitulate the complex multi-system interactions of a whole organism, as is required for 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?

Numbers of animals have been estimated in consultation with statisticians and based historical data from our own experiments using the same tumour models with similar nanomaterials or nanomaterial monitoring approaches.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We aim to use the minimum numbers of animals required to adequately and robustly address the research question. This has been determined with support from statisticians and use of rigorous experimental design considerations (as guided by the NC3Rs Experimental Design Assistant). Use of adequate numbers of animals will reduce variability, improve experimental consistency and confidence in outcomes. All assumptions on which sample size estimates are based will be re-evaluated once additional or new data is available from these studies and if necessary numbers of animals required will be revised for subsequent studies.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The main way we will reduce the number of animals we use will be to use longitudinal monitoring (eg. tumour growth measurements) and live imaging techniques. This will allow us to obtain data from the same animal over time instead of the more traditional method of killing a different animal at every timepoint. Where nanomaterials or nanomaterial therapies are being tested for the first time in animals, pilot studies will be run with smaller numbers to ensure safety and provide an initial assessment of efficacy or effect that will be used to statistically determine the correct number of animals to use for further investigations.

For breeding we will keep this to a minimum to maintain the transgenic line and provide only enough mice for our expected usage within a particular experiment or project. This will be regularly reviewed to prevent the unnecessary breeding and maintenance of genetically modified animals. Finally, at the end of each experiment we will collect as many tissues as possible in order to maximise the potential output from 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.

A proportion of the experiments in this project will use superficial flank tumours. These are the least invasive and have minimal impact on the animal throughout the course of an experiment. The tumours

in these animals can also be visually monitored easily through physical measurement allowing any experiments to be terminated prior to the onset of any systemic clinical signs.

In some cases it will be necessary to use a systemic or metastatic model (eg. when developing a nanotechnology to detect or treat metastasis). For metastatic tumours, while these have more invasive properties they are not usually associated with significant clinical signs. This will be carefully monitored through the use of live imaging and continual (daily or more frequent) health monitoring.

For brain tumours, it is important that the tumour is growing in the brain to mimic what occurs in patients. The site of tumour induction is carefully planned such that tumours are induced in a site of the brain that is associated with minimal or no side effects associated with tumour growth. This also means that if the tumour is surgically removed, this is unlikely to be associated with any additional clinical signs.

In all models animals will be humanely killed before the onset of clinical signs that would exceed moderate suffering.

For administration of nanomaterials, the least invasive route that is relevant for the particular application will always be used. Where more invasive routes are necessary (eg. surgical administration) this will be scientifically justified and through proper aseptic technique, pain management and careful monitoring is not expected to cause any additional distress or prolonged suffering.

Why can’t you use animals that are less sentient?

The animals proposed are the least sentient mammalian species. The use of non-mammalian species (eg. Xenopus, Danio) would not be appropriate for the cancers or the clinical translation of the nanotechnologies under development. Mice and rats are the most appropriate for the work being carried out as they have circulatory, nervous and excretory systems very similar to humans, which allows us to model where the materials go, how the body breaks them down and how they are removed from the body in a system similar to humans. Importantly, the species used have well established cancer models that closely mimic human disease.

Adult animals are primarily used as they have the most complete development of the different systems that our nanotechnologies will interact with, including the brain, cardiovascular system, and immune system. Furthermore the cancers we are investigating are largely those that develop in adults such that it would not be clinically relevant to investigate these in more immature life stages.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals undergoing surgical procedures will receive appropriate analgesia to prevent any post- operative pain, will be carefully maintained at a suitable depth of anaesthesia and may also receive additional fluid support to prevent dehydration associated with longer procedures. These animals will be provided with additional husbandry such as mash/wet food, heated housing and careful monitoring in the immediate hours following surgery until normal activity is resumed.

Animals will be group housed and where animals have been individually housed for a particular purpose (post-surgical recovery) these will be grouped as soon as is appropriate.

Through our previous work we have refined animal monitoring approaches to minimise the harms to animals. Animals with more rapidly progressive disease (eg. later stages of cancer growth) will undergo frequent monitoring (up to twice daily) to identify and humanely kill any animals that may approach humane endpoints before the next monitoring timepoint.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All experiments will be planned and executed with reference to the PREPARE and ARRIVE 2.0 guidelines to ensure effective experimental planning and proper reporting of experiments respectively. We will follow guidance from BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinements and LASA guiding principles for Administration of Substances and Aseptic surgery.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The researchers working under this licence will be regularly encouraged to actively stay informed on advances in the 3Rs as is required by the conditions of their PIL. We will regularly check information on NC3Rs website and newsletters and we will attend institutional and regional 3Rs symposia. Any relevant advances, for example refinement of techniques or approaches, will be readily implemented into this project.

52. Nanotechnology and nanomedicine for detection and treatment of brain disorders

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Nanotechnology, Nanomaterials, Electrophysiology, Epilepsy, Parkinson's disease

Animal types	Life stages
Rats	adult, juvenile
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?

Nanomaterials are very small materials with at least one dimension (eg. width, length, height) typically less than 100 nanometres (one billionth of a metre) in size. The overall goal of this project is to design and test novel nanomaterials or nanomaterial enabled devices for research and medical applications in brain disorders including epilepsy and Parkinson's disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The use of novel technologies based on nanomaterials (nanotechnologies) seeks to design smarter solutions with potential to overcome key clinical problems. Metal-bases devices used to record brain activity are extremely useful, however they do have certain limitations. Novel nanomaterials, such as graphene are capable of overcoming the short

comings of metals, and promise to significantly improve detection of pathological brain activity, provide advanced diagnostic clinical tools, and allow more effective neurostimulation therapy. Several neurological disorders can become drug resistant (i.e. epilepsy, Parkinson's disease), and therefore development of novel therapies, based on either targeted neurostimulation of brain centres or circuits, novel nanomedicines, or personalised gene therapies are warranted to treat these patient populations.

What outputs do you think you will see at the end of this project?

The new information gained from this project will be shared in the form of scientific publications, conference communications and through public engagement activities throughout this project. While the timescale for these nanotechnologies to move from preclinical investigation to clinical products are more likely to be in the long term, we aim to build up rationale and justification within this project for clinical testing and validation of these technologies.

Who or what will benefit from these outputs, and how?

These communications will enable collaboration and stimulate further research into advanced biomedical nanotechnologies throughout the scientific community.

Eventually, patients will benefit from any effective new technologies for the diagnosis, monitoring or treatment of brain disorders that are based on the results obtained during this project.

How will you look to maximise the outputs of this work?

Publication and communication of our findings will always be the primary aim of this work. The goal of every experiment conducted under this licence will be to generate valid, high quality and therefore publishable results and we will endeavour to ensure that all findings meeting these criteria will be published (majority in open access journals) to inform the wider scientific community, even if these do not support the therapeutic, diagnostic or monitoring potential of the particular nanomaterial, or nanomaterial device being investigated.

Species and numbers of animals expected to be used

- Mice: 2450
- Rats: 1650

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 chosen for many of these studies for a variety of reasons, primarily as they are the least sentient mammalian species that will provide data applicable to humans. The disease models using these are already well characterised and validated with close similarities to the human disease such that the findings and nanotechnologies developed have the best chance of clinical translation and population benefit. Rats are used as an

alternative rodent model for some of this work where either the models are more established and refined (Parkinson's disease) or where the larger size of these rodents is necessary for the nanotechnologies being developed (e.g. recording/stimulating devices).

The majority of research will use adult animals as the diseases being investigated are those which primarily affect adults.

Typically, what will be done to an animal used in your project?

Animals will first undergo a procedure to induce the specific disease in the brain.

For Parkinson's disease this may be surgery (injection of a neurotoxin into a discrete region of the brain) to induce a hemiparkinsonian state (affecting one side of the body). After induction of Parkinson's disease, which will be confirmed using a combination of behavioural tests, animals may undergo an additional surgery to implant a nanomaterial enabled device for electrophysiology recording, and/or stimulation. This may be on the surface of the brain, or implanted into deep brain structures similar to deep brain stimulation probes which are already used clinically. The therapeutic effects of nanomaterial device based deep brain stimulation will be assessed by measuring electrophysiology biomarkers of Parkinson's disease, or through behavioural tests to identify any improvement in symptoms. In some experiments, rather than stimulation, animals may be administered nanomaterial-based therapies for Parkinson's disease and either behavioural tests, electrophysiology measurements, or a combination will be used to determine their effectiveness. At the end of the experiment, animals will be humanely killed and tissues collected for further analysis.

For epilepsy, animals will undergo a procedure to induce epilepsy which will usually be injection of chemicals to induce acute seizures or acquired epilepsy, or by implantation of cancer cells in a region of the brain that is prone to seizures (to model tumour-associated epilepsy). Animals will then be implanted with devices (usually nanomaterial enabled) for electrographic recording of seizure activity and spread. This will be used to determine the effectiveness of novel nanomedicines aiming to prevent seizures or reduce the negative impact these have on the brain, or to evaluate the impact of brain hyperexcitability and its effects on tumour growth. At the end of the experiment, animals will be humanely killed and tissues collected for further analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

From our previous work, we don't expect the nanomaterials we use to have any particularly strong adverse effects. Where unexpected or substantial reactions occur, animals will be humanely killed as these effects would likely interfere with the aims of the studies. The procedures used to administer the nanomaterials are usually the least invasive possible. Where surgical administration is needed, this will be done under anaesthesia and analgesia or additional support is provided to minimise the adverse effects associated with this.

For Parkinson's disease the 6-OHDA model of hemi-Parkinson's (our favoured model) is well tolerated. After approximately 12 weeks post-6OHDA administration, rats may show a mild rotational behaviour in the home cage, but this does not affect their wellbeing. Other mild locomotor disturbances including a shuffling gait and short strides have been reported in the literature but are infrequently observed in our studies.

For epilepsy we try to minimise suffering at all times, but in order test the efficacy of our nanotechnologies and nanomedicine it is necessary for the animals to experience the sort of seizures we wish to detect and treat in patients. Patients report that the seizures' themselves are not painful, but they do report post-seizure fatigue and they can injure themselves by falling etc. We will develop treatments to reduce seizure burden in the treated group, this does mean however that the control animals will continue to experience seizures. For this reason, we will choose a study duration of minimal length required to extract the information needed, usually less than 12 weeks. In models of acquired epilepsy some weight loss (~5-10%) is usually observed for the first couple of days after induction; but this is transient and the animals soon regain weight.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice: 100% moderate

Rats: 100% moderate

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Before materials or devices are tested in animals, they will first be tested in cell cultures, or in relevant in vitro systems to check the safety and to help determine the doses that would be safe, or provide a particular effect in vivo.

For brain disorders such as Parkinson's disease and epilepsy, and tumour associated epilepsy; which are disorders of the whole brain, it is necessary to study the whole organ with intact neural networks as well as the connection with the bloodstream, lymphatics and other systems. As well as this, behavioural changes are an important measure of how effective a treatment or stimulation device is, which can only be done in animals.

Which non-animal alternatives did you consider for use in this project?

In vitro cell cultures including 3D models and co-culture systems (up to and including organoids which are 3D in vitro models that contain multiple cell types and mimic organs more closely).

Why were they not suitable?

Non-animal alternatives such as those listed above can provide important information and are always used in the first instance for all new technologies and nanomaterials. This includes testing in organoid systems which is an ongoing effort by our group. However, none of these systems (including organoids) can effectively recapitulate the complex multi-system interactions of a whole organism, or the complete neural networks of the brain, as is required for 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?

Numbers of animals have been estimated in consultation with statisticians using historical data from our own experiments with the same models and approaches with similar nanomaterials or nanomaterial devices.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We aim to use the minimum numbers of animals required to adequately and robustly address the research question. This has been determined with support from statisticians and use of rigorous experimental design considerations (as guided by the NC3Rs Experimental Design Assistant). Use of adequate numbers of animals will reduce variability, improve experimental consistency and confidence in outcomes. All assumptions on which sample size estimates are based will be re-evaluated once additional or new data is available from these studies and if necessary numbers of animals required will be revised for subsequent studies.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The main way we will reduce the number of animals we use will be to use longitudinal monitoring techniques. By implanting recording devices in animals, we will be able to measure electrophysiological biomarkers of the brain disorders overtime and in response to treatment. This will allow us to obtain data from the same animal over time instead of the more traditional method of killing a different animal at every timepoint. Where possible we will use animals as their own control (eg. comparing between stimulation and unstimulated in the same animal). Finally, at the end of each experiment we will collect as many tissues as possible in order to maximise the potential output from 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.

Toxin-induced models of Parkinson's disease (6-OHDA and LPS) will be used to assess the therapeutic efficacy, diagnostic or monitoring potential of novel nanotechnologies. Both these models are well characterised and reproducible model with the animals showing mild symptoms which do not interfere with daily movement or activities. The reproducibility and consistency of these moderate severity models of Parkinson's disease will enable us to reduce the number of animals needed per study but also improve the quality of data obtained both at the biological and behavioural levels.

For epilepsy we will use a range of models in order to select the one that is most appropriate for the nanomaterial, or nanomaterial device being developed. The majority of seizures are generally mild, non-convulsive, and last less than 2 minutes. These models are reproducible and can be modulated by the use of anticonvulsant medication should the initial phenotype be more substantial than is required for the output of the experiment.

Where we are assessing the effect of brain tumour associated epilepsy, cancer cells are administered to sites of the brain known to be susceptible to seizure induction during the growth of cancer. Other than the generation of seizures, which are generally mild, animals show minimal clinical signs.

Why can't you use animals that are less sentient?

The animals proposed are the least sentient mammalian species. The use of non-mammalian species (eg. Xenopus, Danio) would not be appropriate for the diseases or the clinical translation of the nanotechnologies under development. Mice and rats are the most appropriate for the work being carried out as they have nervous systems very similar to humans, which allows us to effectively model the diseases we are trying to treat or diagnose. The anatomy of the brain in these animals is also more appropriate for the development of devices that may eventually be used in humans.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals undergoing surgical procedures will receive appropriate analgesia to prevent any post- operative pain, will be carefully maintained at a suitable depth of anaesthesia and may also receive additional fluid support to prevent dehydration associated with longer procedures. These animals will be provided with additional husbandry such as mash/wet food, heated housing and careful monitoring in the immediate hours following surgery until normal activity is resumed.

We will work with the animal house staff to ensure that suitable environmental enrichment is provided. Animals will be group housed if possible, however some animals with epilepsy or implanted devices will need to be housed individually.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All experiments will be planned and executed with reference to the PREPARE and ARRIVE 2.0 guidelines to ensure effective experimental planning and proper reporting of experiments respectively. We will follow guidance from BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinements and LASA guiding principles for Administration of Substances and Aseptic surgery.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The researchers working under this licence will be regularly encouraged to actively stay informed on advances in the 3Rs as is required by the conditions of their PIL. We will regularly check information on NC3Rs website and newsletters and we will attend institutional and regional 3Rs symposia. Any relevant advances, for example refinement of techniques or approaches, will be readily implemented into this project.

53. Assessing the impact of autoimmune disease associated fibroblasts on anti-tumour 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, Autoimmunity, Fibroblasts, Immunosuppression, Cellular plasticity

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?

Fibroblasts are structural cells within tissues that have been shown to suppress the host immune response to cancer but strengthen it in autoimmune disease. We are aiming to understand whether fibroblasts from individuals with an autoimmune disease have the capacity to strengthen the anti- tumour immune response.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Immune checkpoint inhibitors have revolutionised the treatment for certain types of cancers such as melanoma, non-small cell lung cancer and renal cell carcinoma. Despite their promise, these effects are not universal for all cancer patients, and it is vital to understand how to increase the number of people who will benefit.

The ecosystem around the tumour cells can often suppress the immune response to cancer – this includes fibroblasts, which are important structural cells that can also modulate the immune system. Several cancer-associated fibroblast subtypes have been shown to suppress the anti-tumour immune response, leading to a lack of immune cells in the vicinity of the tumour.

In contrast to cancer, autoimmune diseases such as rheumatoid arthritis and inflammatory bowel diseases have an overactive immune system which causes the recruitment of immune cells to the affected parts of the body. Like cancer, fibroblast populations can drive this effect, but in this case promote the host immune response to healthy tissue.

We are interested to understand whether fibroblasts from an autoimmune disease setting may increase the number of immune cells in the vicinity of tumour cells, and whether this also translates to a subsequent shrinkage of tumours.

If autoimmune disease fibroblasts can do this, it may be possible to use the mechanisms employed by these cells to enhance current cancer immunotherapies, and perhaps as a treatment.

More broadly, this work will help us better understand fibroblasts and their capabilities across both cancer and autoimmune disease, facilitating our ability to harness these cells for therapeutic benefit.

What outputs do you think you will see at the end of this project?

Advances in scientific knowledge on the factors and molecular mechanisms underlying fibroblast immunoregulatory function across cancer and autoimmune diseases.

- Scientific and lay publications of our findings and methodology.
- Presentation of our data and/or methodology to the wider scientific community through conference oral and poster presentations.
- Identification of novel therapy targets or agents which can be taken forward to develop new therapies.
- Generation of cell sequencing data from these studies that will be made publicly available to the scientific community.

Who or what will benefit from these outputs, and how?

This project will provide a unique opportunity to combine knowledge of the biology of fibroblasts across the two interrelated fields of cancer and autoimmune disease, leading to benefits for several stakeholders:

1. Cancer researchers

These experiments will help cancer researchers understand whether fibroblasts from an autoimmune disease setting are able to augment the anti-tumour immune response. If this is shown, the mechanisms employed by these fibroblasts may be harnessed for potential immune enhancing treatments within the tumour microenvironment. On the other hand, if this is not demonstrated, these experiments will also provide insight as to why these

immune stimulatory fibroblasts do not function in a similar manner within the cancer context. Identification of the contributory factors may be investigated further to augment the efficacy of cancer immunotherapy.

2. Fibroblast biologists

Different populations of fibroblasts have distinct immunoregulatory roles in different tissues and diseases. These experiments will provide fibroblast biologists with further insights as to whether these effects can be maintained in a completely different setting, or whether factors within the individual tissue or disease context ultimately determine the immunoregulatory function of fibroblasts. This knowledge will guide strategies to modulate fibroblast behaviour across diseases.

3. Autoimmune disease researchers

Fibroblast subpopulations have been demonstrated to be key drivers of the inflammatory processes in autoimmune diseases such as rheumatoid arthritis, and there is great hope that these cells can be targeted for therapeutic benefit. If it is the case that fibroblast immunostimulatory function is reversed by signals from the tumour microenvironment, this will help to identify new pathways that can lead to the resolution of inflammation by re-programming these immunostimulatory fibroblasts to be immune suppressive. Autoimmune disease researchers can then test the utility of these tumour-derived factors across a range of inflammatory disease settings.

4. Patients with cancer or autoimmune disease

The ultimate goal of this project is to develop better treatments for patients with cancer or autoimmune diseases. A better understanding of fibroblast biology is fundamental to realise this aim.

How will you look to maximise the outputs of this work?

I will disseminate my research outcomes to the research community via scientific publications and presentations at conferences. In terms of publications, we aim to publish high impact papers based on the findings generated from the research grants funding this project licence. In addition, our group has a strong tradition of publishing methodology papers and negative data to ensure that groups do not unnecessarily repeat experiments that either technically are flawed or biologically yield the null hypothesis. I will also aim to share my findings every year at a major scientific conference either locally or internationally. This will allow me to engage an international group of experts including basic scientists and clinicians, giving me the opportunity to gather feedback to further refine my research.

I will also be presenting my work more regularly during lab meetings and during meetings with peers and more senior members of the organisation, allowing for a consistently high level of scrutiny of the project.

It is important to engage patients throughout my research. I have already begun this process by presenting my project proposal to patient interest groups. I will continue to have regular meetings over the course of this project, and they will be able to help with the organisation of events to promote my research to the wider public.

Social media is increasingly important in the communication of scientific research. Therefore, I plan to use such platforms to publicise my work in addition, and to engage with interested parties virtually via this medium.

Species and numbers of animals expected to be used

- Mice: 640

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are the best vertebrate model for studying the immune response to cancer because:

1. mice are the least sentient species that share sufficient immune system similarities to humans - this is essential as immune responses as opposed to the function of individual genes are being studied.

2. an extensive range of reagents are available for analysis of the cellular and molecular interactions occurring during immune responses.

Adult mice will be used for the subcutaneous injection of tumour cells and fibroblasts to ensure a mature immune system. Additionally, the disease processes we are studying operate in adult human disease. This is also the standard age of mice used by groups who employ similar tumour models, thus avoiding the need to use additional animals to obtain equivalent data.

Typically, what will be done to an animal used in your project?

To generate murine tumour models, mice will be injected subcutaneously in the flank with either tumour cells alone or tumour cells mixed with fibroblasts. Cells will be suspended for injection in an appropriate vehicle such as phosphate buffered saline / culture medium or in Matrigel.

The mice will be observed post injection to ensure there are no complications. Appropriate anaesthetics and post-implantation analgesia will be used as required.

Measurement of the tumour size in three dimensions using calipers will begin as soon as the tumour is palpable. The body condition score of the mice will be recorded on the day of injection and regularly throughout to aid with welfare monitoring. Mice will be checked everyday if the tumour diameter approaches the maximum of 12mm in any dimension, or a volume of 1.25 cm3. The volume of the tumour will be calculated using the following formula: volume = (length x width x height) x 0.52. This equation has been shown to best represent the mass of a wide variety of tumour shapes and sizes.

Mice will be humanely killed at the end of the experiment and the tumour will be removed for analysis. Whilst part of an experimental cohort, animals may be given substances via subcutaneous injection, for the purpose of imaging the tumour growth. Cohorts of mice will receive immune checkpoint targeting treatments relevant to clinical practice (typically by

intraperitoneal injection to enhance bioavailability of these agents) to assess the effect of the fibroblasts on these therapies. Furthermore, treatments with reagents targeting fibroblast-derived signals or cells in communication with the implanted fibroblasts will be given to dissect the effector mechanism of the fibroblasts and to validate findings from the single-cell RNA-sequencing analysis of inflammatory synovial fibroblast-rich tumours. Based on prior literature, examples of likely interventions include inhibition of IL-6 with a neutralising antibody and antibody-mediated depletion of T cells. Blood samples may be taken according to recommended published guidelines (on a weekly basis) and/or a larger final sample under terminal anaesthesia.

In order to generate inflammatory activated joint tissue fibroblasts (also known as synovial fibroblasts), we will induce arthritis and mice will be humanely killed at various time points over the course of arthritis and their joints harvested for the isolation of synovial tissue fibroblasts for use in the tumour implantation model. It is necessary for the mice to develop advanced arthritis given that synovial fibroblasts from this setting have been previously shown to increase inflammation, and are thus the most likely to augment anti-tumour immunity. During arthritis animals will undergo daily handling for scoring and calliper measurements of joints and limbs; administration of an agent to induce arthritis and will be killed up to week 8 via a schedule 1 method.

What are the expected impacts and/or adverse effects for the animals during your project?

The maximum severity limit in this licence is moderate.

For tumour implantation: animals may experience pain and discomfort from implantation of cells and the administration of substances, but the discomfort is expected to be minimal and not long-lasting. Mice tolerate subcutaneous tumours well. Tumours can rarely ulcerate if rapidly growing or if low on the flank can interfere with movement. If mice develop these adverse effects they will be humanely killed.

Any animal showing deviation from normal behaviour as judged by body weight, body condition, general and coat appearance, gait or behaviour will be further monitored. Before adverse effects exceed a moderate severity level, animals will be killed to prevent any ongoing pain or suffering.

For arthritis: possible adverse effects include irritation from local injection, pain from joint inflammation, weight loss or mobility problems. Any animal showing deviation from normal behaviour as judged by daily monitoring of food and water intake, body weight, general and coat appearance, gait or behaviour will be treated with pain relief and food supplements. Before pain and inflammation exceed a moderate severity level, animals will be killed to prevent any on-going pain or suffering.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Moderate 100% - All animals are expected to have moderate category severities given that tumours will be induced subcutaneously, and that these are expected to cause mild pain

or distress or moderate interference with normal behaviour. Arthritis is also moderate severity as it can cause pain and distress and interfere with normal mobility.

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 key question is whether inflammatory fibroblasts from an autoimmune disease setting can affect the anti-tumour immune response. Despite advancements in in vitro techniques, these are unable to fully recapitulate the physiological immune response to cancer. This is best modelled in an in vivo setting; in a model system whose immune system shares sufficient similarities as humans.

Additionally, the autoimmune disease fibroblasts we will use for our study do not normally interact with cancer cells anatomically in vivo, therefore to study this scenario there is no choice but to use an animal model in which we can perform implantations of mixed cell populations. The proof-of-concept nature of this study makes undertaking this intervention in human cancer patients unethical.

Which non-animal alternatives did you consider for use in this project?

We considered a range of co-culture experiments of fibroblasts and cancer cells. While these are unsuitable to assess the impact of fibroblasts on the anti-tumour immune response, a mixed cell spheroid system will help to determine the optimal ratio of tumour cells and fibroblasts for implantation into the in vivo models.

Why were they not suitable?

While the in vitro models are useful to inform how tumour cells and fibroblasts interact, these do not fully recapitulate the complex tumour microenvironment as well as the anti-tumour immune response which consists of multiple effectors.

Furthermore, phenotypic changes as assessed by techniques such as flow cytometry and gene expression do not fully correlate with the immunological effect of fibroblasts in vivo. For example, fibroblast subtypes which are immunosuppressive in cancer appear to be immuno-stimulatory in inflammatory arthritis. Additionally, several of the signals secreted by fibroblasts have different immunological effects depending on the tissue context. Such nuances will not be captured by in vitro models.

Nevertheless, there will be a role for these techniques. For example, tumour cell/fibroblast co-culture experiments will be performed prior to the in vivo experiments to optimise experimental conditions needed in mice - such as cell numbers needed for implantation. In summary, these in vitro experiments will supplement our animal experiments and therefore reduce the overall number of mice needed.

In order to generate activated synovial tissue fibroblasts for implantation it is necessary to use freshly isolated fibroblasts from the joints of mice with arthritis, otherwise fibroblasts rapidly lose their phenotype in vitro and there are currently no validated methods for maintaining their phenotype in vitro prior to transfer to an in vivo setting.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

For all experimentation, the lowest possible number of animals will be used whilst ensuring that the experimental result is robust. The NC3Rs Experimental Design Assistant was used to inform the number of animals used per experiment, using an anticipated effect size based on previous experience and published data. The output of this tool was compared with the sample sizes used in similar studies within the literature.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The animal studies have been designed with the NC3Rs (National Centre for the Replacement, Refinement and Reduction of Animals in Research) Experimental Design Assistant (EDA). This tool has given us estimates of the number of animals required to deliver meaningful results for our experiments.

While arthritis induction in mice is needed to generate synovial tissue fibroblasts for tumour implantation studies we will reduce the number of mice needed to generate freshly isolated fibroblasts by using where possible, surplus cells from other experiments being performed.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Where new interventions are being examined, pilot studies will first be established in 2-3 mice prior to full experiments. Subsequently these pilot data will be used in the specific mathematical calculations described above to ensure that we use the minimum number of animals needed to obtain statistically significant results. To maximise the information gained from a single animal, we aim to perform multiple analyses on tissue obtained from each individual animal.

Pilot studies will also be performed to ensure the number of cells injected into the mice is optimal. The mice will then be followed for 60 days to assess for the formation of well-established tumours, with minimal variation between the mice.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the

procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice will be injected subcutaneously with tumour cells alone or together with fibroblasts. This will be an immunocompetent syngeneic model given that the tumour cell lines we will use have also been derived from the same mouse strain, thus facilitating study of the anti-tumour immune response. All tumour

cells will be checked for mycoplasma infection to reduce the risk of any animal suffering from infective complications.

Subcutaneous implantation of the tumour will be used as the technique that causes the least distress. First of all, subcutaneous injection only causes momentary discomfort for the animal. Furthermore, injection into the flank minimises the risk of the tumours compressing vital or sensitive parts of the body.

We also wish to investigate how activated fibroblasts behave and contribute when implanted into the tumours. In order to do this, we will use a polyarthritis mouse model to generate these activated fibroblasts. This model involves injecting animals with serum from a genetically altered mouse strain, causing the recipient to develop arthritis. The subsequent activated fibroblasts will then be extracted post-mortem from the synovial tissue. Whilst the mice need to develop established arthritis to ensure the fibroblasts are sufficiently activated, the mice will be maintained for the minimum amount of time possible at this established stage,and will receive appropriate analgesia when needed .

The mice will be monitored closely and regularly, with very clearly defined humane endpoints. Mice will be humanely killed before adverse effects exceed a moderate severity level.

Where possible we will use established reagents and protocols to treat the mice. The lowest doses of agents that are well tolerated and effective will be used. For many drugs there is information available in the literature to guide formulations for in vivo experiments, but pilot studies will be used before starting larger experiments.

We will use both male and female mice in a 1:1 ratio where possible.

Why can’t you use animals that are less sentient?

The immune system of less sentient animals do not fully represent that of humans. Small rodents are the lowest mammals that can be used to recapitulate the response of the human immune system to a localised insult such as a tumour. The model requires the use of live animals since our aim is to track changes in the immune response to tumour cells, therefore terminally anaesthetised animals cannot be used.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Tumour induction in mice will be performed in accordance with published UK guidelines (Workman et al., British Journal of Cancer 2010). To minimise distress, the tumour will be implanted subcutaneously into the flank, a site that does not affect normal body functions such as eating, drinking, defecation, urination and ambulation.

Mice with developing tumours will be monitored until a palpable tumour is felt. Frequency of monitoring will be increased at this point for effects such as rapid tumour growth, ulceration and in consultation with the NVS. Other clinical signs to be observed include general appearance, weight loss, dehydration, abnormal activity, posture, state of hair coat, or any interference with vital physiological function.

The size of tumours will not be allowed to exceed 12mm or ulcerate – if so, the mouse will be humanely killed. Furthermore, if the mice display limitations in their health such as restriction in mobility, weight loss, or a body condition score of 2 or less, the mice will be humanely killed.

The methods required to induce the polyarthritis model have been refined in our laboratory, and thus we will be able to use the lowest doses of the necessary agents to achieve the required degree of arthritis whilst avoiding unnecessary pain, suffering and distress. In addition, our group is experienced with the monitoring the humane endpoints of this model and assessment of arthritis severity using established methods.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Animal welfare is a key consideration in all of our protocols, and we will be guided by our NACWOs and NVS in always ensuring that we are using best practice and the most refined techniques. All staff involved in animal experiments will review the literature on animal welfare provided by the local AWERB. Following every experiment and regularly during group meetings we will review our procedures from a welfare standpoint to identify any potential for refinement.

Relevant guidance on the welfare and use of animals in cancer research has been published (Workman et al., British Journal of Cancer 2010), and we will perform our experiments in accordance with these guidelines. We will also refer to published literature on arthritis models, such the Working Group report by Hawkins et al., Inflammopharmacology 2015), which describes the methods of applying refinement to the use of mice and rats in rheumatoid arthritis research.

Additionally, we will follow the PREPARE and ARRIVE 2.0 guidelines for our planning and reporting of our experimental findings.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I or a member of my research team will attend relevant NC3Rs workshops/webinars and sign up to the NC3Rs newsletter. We will also be reviewing the literature on regular basis in our journal clubs and through this network we will discuss any refinements that could be applied to our own work.

54. Assessing the efficacy of virus-mediated gene delivery to the central nervous system

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Neurodegeneration, gene therapy, viral vectors, nerve regeneration, brain

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to establish the efficacy of viral vectors to not only target specific neuronal populations in the brain but also to assess modulation of therapeutic gene targets that are linked to neurodegenerative conditions.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

It is estimated that one in six people in the UK have at least one neurological condition, with an estimated 600,000 new cases diagnosed every year. Brain disorders such as amyotrophic lateral sclerosis (ALS), Alzheimer’s disease (AD), Parkinson’s disease (PD), Huntington’s disease (HD) and frontotemporal dementia (FTD) cost the UK economy £112 billion a year (direct medical and indirect costs such as lost time at work). Currently, there are no treatments for these diseases which can reverse the damage caused and only palliative treatments to manage their symptoms are available, leaving an urgent medical

need for effective therapies. It is recognised however, that therapies which promote neuronal survival and nerve regeneration might be one way of reversing the loss of function that occurs. In this project, we wish to test therapeutic targets, delivered by gene therapy into the brain and measure how many neurons and supporting cells are infected by the virus and ultimately determine cell specific changes in appropriate target molecules.

For most central nervous system (CNS) diseases, gene delivery by direct injection into specific areas of the brain may be the most efficacious way of delivering a therapy. For example, we plan to inject the brain stem, the midbrain, the hippocampus/substantia nigra and the basal ganglia/cerebral cortex to target specific neurons responsible for causing these conditions. These have been determined by others in published studies as sites where neurons in those areas are specifically affected by each of these neurodegenerative diseases. This will ensure specific neurons associated with each of the neurodegenerative diseases are targeted efficiently. We also need to vary the dose of the gene therapy system so that we can achieve variable levels of gene alterations. For example, when it comes to gene silencing, most people aim for >70% change in the target cell or area. However, we have shown using small molecule drugs that even a 30-50% change can have significant benefits. Hence, we will titrate the dose to achieve 30-50% change in the appropriate genes in some case. In other cases, we will aim for 70% gene changes in the target area.

The advantage of viral delivery systems over small molecule drugs is that these can deliver the appropriate therapeutic gene for at least nine months or more after a single injection. This circumvents the need for repeated injections, as well as avoiding the natural barriers in the brain that prevent entry of many drugs into the CNS via other injection routes such as intravenous or intraperitoneal delivery.

What outputs do you think you will see at the end of this project?

The primary outputs of this work will be new knowledge on the efficacy of gene therapy to deliver therapeutic genes into the CNS, and in particularly the brain. We wish to understand how many neurons and other cells are infected by the virus-based delivery system and the levels of gene modulation in whole tissue lysates and individual cells in the normal brain. These parameters will affect the overall efficacy of our gene therapy approach and help us to fine tune our therapy for future studies in mouse models of neurodegeneration.

Specific identifying outputs will be to publish the findings. This is important for the development of the project but also to provide a knowledge base for other academics working in this field.

Other product outputs will be to support the development of the new and existing intellectual property which will allow us to facilitate translation into the clinic.

Who or what will benefit from these outputs, and how?

From our other work in the laboratory, we have identified several target molecules that promote regeneration of axons in the CNS after injury and are also affected in neurodegenerative diseases. We now need an appropriate delivery system to either up- or down-regulate specific target molecules, without requiring repeated injections into the brain. In the short term, the benefits would be to provide new knowledge to fine tune the delivery of therapeutic genes into the CNS for future studies in disease models.

In the longer term this work has the potential to optimise the delivery of already identified therapeutic molecules by gene therapy into the CNS and to offer potential treatments to be tested in mouse models of neurodegeneration. There is currently no treatment that reverses the pathological effects of neurodegenerative disease and as a result permanent loss of function occurs. Our optimised gene delivery systems could provide vital treatment strategies to reverse or protect against the damaging effects of neurodegenerative disease.

Examples of other potential beneficiaries of the success of this work are people with traumatic injuries such as traumatic brain injury, traumatic spinal cord injury and stroke, who also require the same molecules to reverse the damage to axons that is caused by injury and hence will benefit from this work as the knowledge can be transferred to these systems also.

The UK economy spends £112 billion treating brain neurodegenerative diseases every year and represents a significant financial and personal burden. There are currently no preventative measures available for neurodegeneration but age is a risk factor and often the exact cause is unknown.
Therefore, effective therapies that reverse the damage to the brain from neurodegeneration and promote its repair and regeneration, would relieve the pressure on the NHS and significantly reduce the personal and societal costs of treating neurodegeneration.

How will you look to maximise the outputs of this work?

We will maximise the outputs of the work by collaborating with colleagues and external collaborators working in this field to maximise the use of the data we obtain. We will rapidly disseminate the outcomes of the research, whether negative or positive, to inform the academic community and support other researchers developing technologies in this area. We will seek partnerships with appropriate external collaborators when our current therapies have been optimised, giving us the potential to translate our therapies into the clinic for patient benefit.

Species and numbers of animals expected to be used

- Mice: 300

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

For all of experiments, we will use wild type adult mice since this is the least sentient animal to achieve our scientific aims. We have chosen adult mice because our proposed target therapies will eventually be evaluated in neurodegenerative disease models in adult mice, if our route of delivery is shown to be successful. Adults are also used since age is a critical predictor of disease. No disease models will be used in this project as we are optimising the gene delivery parameters and determining levels of gene changes prior to experiments in neurodegenerative models. In addition, most neurodegenerative models

we want to study in the future have been developed in mice since genetically altering mice is significantly easier than in rats for example. Continuity in the same species will be key to making timely progress. We cannot use lower sentient species like zebrafish as they regenerate their brain spontaneously and are not suitable. As in the human, the mouse CNS has a poor intrinsic capacity to spontaneously repair and regenerate its central nervous system and hence is invaluable in finding new therapies to prevent neurodegeneration.

Typically, what will be done to an animal used in your project?

Under anaesthesia, a burr hole will be made into the skull, followed by a single stereotactic injection of adeno associated virus (AAV), carrying genes for the pathway of interest and a fluorescent tag, into discrete areas of the brain including the cortex, hippocampus or cerebellum. Animals will then be allowed to recover and humanely killed at various time points to analyse the success of gene delivery and target gene modulation. Brain tissue will be collected post-mortem and subjected to a host of tests including histology, protein and mRNA analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

Based on previous experience of performing injections into discrete areas of the brain, we expect the proposed injections to be well tolerated with no discernible physiological changes post-recovery. Post- surgical pain is managed via appropriate use of analgesia.

We have extensive experience of these injections and have refined our technique to ensure precise needle placement and minimal leakage of injection material.

The therapeutic targets themselves are not expected to cause any adverse effects based on our experience of using them in other neurological models in our laboratory.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Animals will typically experience a moderate severity procedure as they will receive an anaesthetic event followed by a creation of a burr hole through the skull and a single injection of AAV carrying a reporter gene or AAV carrying a reporter gene and a therapeutic gene or AAV and reporter and control gene.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.
Why do you need to use animals to achieve the aim of your project?

Whilst some elements of the CNS can be modelled in dissociated cells or brain slice cultures, the complex, clinical picture and interaction of the whole-body systems, including in particular the immune and nervous systems cannot all be currently modelled in cell-culture or computer-based models. The use of live animals is therefore unavoidable and essential for discovery science, and to demonstrate the activity of potential gene therapies in a situation relevant to the human condition. Neurons are not present outside the animal kingdom and so an animal is required. Only mammals have a sufficiently developed immune-system to readily compare to humans, and rodents are the animals of lowest neurophysiological-sensitivity required to achieve the scientific aims. Zebrafish or other lower sentient animals cannot be used since these species spontaneously repair damage to their brains and are therefore not representative of the human condition. Therefore, there is no feasible alternative that can entirely replace the use of a living animal that would allow our objectives to be met. However, we will use in vitro and ex vivo work to inform our animal studies.

Which non-animal alternatives did you consider for use in this project?

There are currently no alternatives to animal work for brain neurodegeneration models. Cortical organoid models are currently in development and hence their use was also considered.

No cell culture-based model exist that encompass all of the aspects of disease for any of the models described in this project. However, individual aspects will be modelled in vitro and ex vivo. For example, we regularly use in vitro cell cultures to detect AAV serotype tropism to specific neuronal populations we want to target. We also pre-optimise target gene modification, either knock down or upregulation in neuronal cultures and determine their beneficial effects in culture in terms of neuroprotection and axon regeneration, prior to in vivo use.

Why were they not suitable?

Cell culture-based models are not suitable since they cannot encompass all aspects of a fully functional CNS, which comprises interactions of various neuronal and non-neuronal cells as well as the natural barriers that exist within the brain, which limit drug entry.

Cortical organoid models are incredibly difficult to establish as they rely on taking skin cells and then converting them to neurons using a concoction of growth factors, in growing conditions that are difficult to regulate. In addition, the process of establishing cortical organoids converts the cells into an immature phenotype and hence they are ideal for investigating neurodevelopmental disorders but not neurodegeneration that occurs in adults.

The fundamental reason why the use of animals is required is that to understand the complex processes which occur in the brain, which no in vitro method at present can model. It is almost impossible to use primary cells to culture all of the different types of cells in the brain since they require different growth mediums and factors for survival. Indeed, the reason why many new drugs fail between cell culture and in vivo studies is in the inability to full recapitulate the in vivo environment.

Technologies are being developed to address this gap, including the development of 3D cultures. However, none of these model systems are yet able to phenocopy the integration

and interplay between the numerous cell types that constitute the whole CNS environment.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals to be used in this project have been based on pilot data, in-house data and published studies.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We used the NC3Rs EDA system to calculate animal numbers to be used for this project with calculations setup to achieve a statistical significance of α $p<0.05$ with a power of 0.8. Whenever possible, we used our own published data to feed into the EDA system to generate 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 seek to refine protocols, such as the development of other quantitative measures for assessing efficacy such as associated biomarkers that can predict efficacy. Experiments will be planned so that they can be published in accordance with the ARRIVE 2.0 guidelines.

Wherever possible, we will use archived samples and share control groups across different experiments.

As part of good laboratory practice, we will write a protocol for each experiment including: a statement of the objective(s); a description of the experiment, covering such matters as the experimental treatments, the size of the experiment (number of groups, number of animals/group), and the experimental material; and an outline of the method of analysis of the results (which may include a sketch of the analysis of variance, an indication of the tabular form in which the results will be shown, and some account of the tests of significance to be made and the treatment differences that are to be estimated). We will make appropriate arrangements to randomly assign animals to experimental groups and blind studies to reduce the risk of bias.

At the end of the experiment, we will harvest the maximal possible number of tissues and biofluids. Tissues not immediately analysed will be archived and will be made available to other researchers working on similar questions.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use wild-type mice to assess the efficacy of our gene therapy agents. Mice are being used to optimise the gene therapy systems since neurodegenerative models in future studies have been established in the mouse and are well studies. Animals do not show overt outward signs after direct injections into the brain, since we use small volumes and stereotactic apparatus to precisely guide our injections. Injections into the brain have been refined in my laboratory for over 20 years, using similar viruses and genes, without any signs of pain, suffering or distress. There should be no lasting harm in response to the brain injections but there may be some transient post-surgical discomfort that will be managed by appropriate use of analgesia.

Why can’t you use animals that are less sentient?

We cannot use less sentient species (e.g. zebrafish) for this work, because unlike mammals, they are able to repair damage to the brain spontaneously. Mice will be used for our experiments since they share similar pathophysiology to humans with neurodegenerative conditions. Some in the field have argued for larger animal models to be used such as rabbit, sheep, swine and monkeys, but all of these are scientifically unnecessary since insights into human biology can be achieved using mice. In addition, very little is known about the pathophysiology of neurodegenerative disease in these models compared to the mouse which has received a significant amount of attention. Therefore, our mouse model is the best model for use in optimising gene therapy to the brain.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Stereotactic injection of gene therapy vectors into discrete areas of the brain is the most refined method, since it is precise and is performed by experienced staff who are well versed in minimising leakage of the vectors. The injections are painless since animals are anaesthetised and there is no change in normal animal behaviours after such injections. Therefore, injections into the brain carries no further adverse events than those for any other surgical technique. Pre-operative and peri-operative analgesia to minimise pain is used as standard, whilst the small injection needle and the injectate volume ensures minimal side-effects.

Whilst we expect no adverse events from injections of agents into the brain, we will nevertheless undertake pilot studies to confirm this prior to performing larger experiments. Some of the potential therapeutic agents can be evaluated and optimised in vitro prior to in vivo application. We use the minimum number of interventions and minimal volumes for gene delivery during experiments and continually seek methods to reduce these by studying alternative gene delivery strategies. All animals are picked up using refined handling techniques to minimise distress and the technical staff are well versed in

recognising the signs of distress in these animals as they have many years' experience in handling and caring for our animals. We use bespoke welfare sheets that include body conditioning scores to monitor animals post-operatively. All of these refinement steps significantly reduce the animal welfare burden.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Prior to all experiments, we will consult the PREPARE guidelines checklist to ensure that valuable data will be generated in the experiment.

Experiments will be conducted in accordance with the guidelines published by the Laboratory Animal Science Association (LASA).

The resulting data will be published in Open Access Journals wherever possible and in accordance with the ARRIVE 2.0 guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay informed of advances in the 3Rs through attendance of seminars and conferences, as well as discussions with the named veterinary surgeon and the named animal care and welfare officer.

We will review each experiment on completion to determine any refinements that can be applied to future experiments.

Continued review of the scientific literature will be undertaken on a regular basis to identify any newly emerging technologies and models that could be potentially adopted to replace in vivo animal use.

We will also stay up to date with guidance published by LASA on the most refined experimental methods. We are already signed up to receive the NC3Rs newsletter and will attend local events such as conferences and follow advice in webinars hosted by NC3Rs.

55. Genetic-microbial interactions at mucosal surfaces

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 cells, Microbiome, Mucosal immunity, Cystic fibrosis

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 is use mouse models of human genetic and infectious diseases affecting the function of internal body barriers in the gut and lung, characterise the microbes at these sites and manipulate them in order to better understand their contribution to disease pathogenesis.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 human microbiome is a term used to describe the bacteria and other microorganisms that live on, and in, the human body. These microorganisms coexist with us. They are with us from birth and they play an important role in shaping the development of our immune system.

Much of the interaction between the microbiome and the immune system occurs at specialised barrier surfaces, such as those found in the gut and lung. These surfaces are

adapted to protect the body from invasion. They also enable human immune cells to interact with both good and potentially harmful microbes and the substances (metabolites) they produce.

Genetic diseases (diseases caused by errors in human DNA sequence) may sometimes result in disruption of normal function at barrier surfaces. This is true of diseases such as cystic fibrosis (CF) and inflammatory bowel disease (IBD), as well as a number of rare genetic conditions. Under these circumstances, the breakdown of normal interactions between the body (in particular the immune system) and its microbiome may contribute significantly to disease development. Understanding the contribution of the human microbiome to genetic diseases involving disruption to barrier surfaces is therefore important. It may lead to a better understanding of how these diseases develop and opportunities for new drug development. It may also lead to opportunities to manipulate the microbiome itself as a novel form of treatment.

What outputs do you think you will see at the end of this project?

Outputs may include the identification of both common disease pathways and novel treatment targets. Our research intends to better understand these diseases and the role that microorganisms play in driving pathology, with the aim of designing better treatments that can ameliorate symptoms. A greater understanding of the role of intestinal microbes in disease onset, promotion and protection may also lead to more targeted interventions and design of synthetic microbes as an avenue for treatment. We will also investigate how microorganisms at different sites of the body (lung and gut) can cross- influence the immune response to them. This will give us a better understanding of the wider consequences of treatment with antibiotics for example, and enable us to better assess the risks involved depending on other microorganisms present. All of this work will be published in peer- reviewed publications, and disseminated to the broader scientific community through conferences.

Who or what will benefit from these outputs, and how?

Our programme of work which involves fundamental basic and translational science aims to identify key immune and tissue pathways that control the host environmental interface at barrier surfaces such as the lung and intestine. Our research intends to better understand these diseases and the role that microorganisms play in driving pathology, with the aim of designing better treatments that can ameliorate symptoms for the patients who have them. Some benefits to patients may be within the project lifespan of 5 years, for example having a better understanding of the wider effects of antibiotics or antifungals may lead to changes in when they are used. Other benefits such as better treatments are likely to take longer. Whilst cystic fibrosis affects 1 in every 2,500 births, other genetic diseases may be much less common, and less well understood, and a better understanding of disease mechanisms will benefit these rare patients in the longer term.

This information will be used to design interventions to promote health, which may be translated to human disease through collaborations with industry partners.

Other scientists will also benefit from a greater understanding of the role of the microbiome in diseases that are genetically driven or caused by infectious organisms, as this work will enable them to include the microbiome as another variable in disease models.

How will you look to maximise the outputs of this work?

The outputs from this work will be maximised through the dissemination of our findings in publications and conferences. In addition, we will continue to publish our negative results (when relevant) in the hope of minimising redundant work.

We also have a number of successful collaborations in both academia and industry.

Species and numbers of animals expected to be used

- Mice: 11,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The mouse is the most appropriate species for our studies because the mouse models that we will utilise are the best characterised models of gastrointestinal infection and inflammatory diseases, and they show the main features of human disease whilst being the lowest neurological sensitive species that does so. Most of our experiments will be performed on adult mice, but as microbial colonisation starts at birth and significantly changes in early life, we may alter microbial colonisation in pregnant and juvenile mice.

Typically, what will be done to an animal used in your project?

Many of the animals in this project will have an inflammatory disease. We will use a model of cystic fibrosis which has a correction to avoid any gut symptoms, and the lung symptoms in this model are generally much milder than in human patients. Disease will either occur spontaneously (very mild), or will be induced by the addition of microbial species including bacteria and fungi. These will be given orally to colonise the intestine, or through the nose or directly into the lungs to colonise the lung tissue. Inflammation can take days or weeks to develop, depending on the model, but most experiments will be terminated within 6-8 weeks, although some animals may be kept for longer to assess long term effects and long term stability of microbial colonisation.

Prior to or during inflammation mice may also be injected with other substances such as blocking antibodies, antibiotics, or fluorescent dyes, or may be given antibiotics to deplete the microorganisms present. Some mice will be bred without any microorganisms at all.

What are the expected impacts and/or adverse effects for the animals during your project?

Injections are expected to cause local transient pain, with no longer term effect.

Colonisation with pathogenic microorganisms is expected to cause inflammation at the site (intestine or lung) leading to general discomfort, such as a more tender abdomen. Mice can develop loose faeces, and lose up to 15% of their initial body weight. Mice with specific genetic alterations may exhibit an increased risk of rectal prolapse. For lung infection mice may show an increased breathing rate.

Following onset these symptoms are expected to last 1-2 weeks in most cases. Breeding mice with no microorganisms can cause an increase in the size of the caecum over time, which can lead to blockage of the intestine.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

10% subthreshold, 30% 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?

Lung and intestinal physiology and disease involves complex interactions between host genetic factors and the environment and these cannot be reproduced in vitro. A mammalian species is required to permit the study of the innate and acquired immune mechanisms that contribute to the development and control of mucosal infection and inflammation, and it is not possible to replicate the complexity or dynamics of these host-microbe interactions in vitro. Furthermore, the tissue anatomy in which these host-microbe interactions occur cannot be reproduced in vitro.

Which non-animal alternatives did you consider for use in this project?

We have established a 3D organoid culture model whereby different cellular populations are grown together in a gel matrix to re-create certain parts of the intestinal structure in vitro. This allows us to grow, manipulate and analyse some rare intestinal cell populations in the dish.

We are also using complex bioinformatic tools to better identify the microorganisms that exist in previously published microbiome samples and will use these to generate better overall maps of microorganism genomes.

Patients with alterations in the genes that are of interest are recruited to provide faecal microbiome samples. We will have access to genome sequencing data for these patients as the specific genetic alterations will be critical in our understanding of disease development. We will also use healthy controls, who we will try to age and sex match with patients, and for whom genetic data is likely to be inaccessible. Stool samples will be taken to analyse the microbial composition prior to animal work being undertaken.

Why were they not suitable?

Although we are able to culture immune cells and bacteria in vitro, the conditions and media used to culture mammalian cells and intestinal bacteria are often mutually incompatible, meaning it is not possible to study interactions between them in a dish. Three-dimensional and co-culture systems are not yet advanced enough to replace all animal work.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimated numbers of mice to be used are based on our previous experience of other projects. Due to the role of the microbiome in our disease models it will be important to be able to breed these strains with the correct littermate wild type controls, and we have accounted for this in our breeding estimates.

We have also estimated the statistical power and frequency of our experimental set-ups to calculate the number of mice we will use.

In experiments designed to test whether a particular manipulation has led to a reduction in the severity of disease compared to the control group, the number of animals in each group has been calculated to ensure a 90% chance of detecting a change that is statistically significant at the 5% level. For example for categorical data where the probability of mice in the control group getting disease is 0.8 and where we would like to be able to reliably detect a reduction in the test group to 0.3, power analysis indicates a group size of 20. This group size, taken together with the number of different types of manipulation has been used to estimate the number of animals to be used.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Age and sex-matching within experiments will be used to reduce inter and intra-group variation, thus reducing the numbers required to generate statistically meaningful results. We will perform frequent monitoring of the microbiome of each strain to minimise variability due to unintentional microbial changes. We make use of the NC3R's Experimental Design Assistant, G*Power2 and other online tools to assist with calculating appropriate experimental group sizes. If experiments are more complicated and require more specialist input we will seek support from statisticians local to our institute, and within our wider consortium, which contains multiple experienced bioinformaticians and statisticians.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Every effort will be made to reduce the number of mice as follows:

- Use of pilot experiments with up to 5 animals per group for dose determination or model development

- Shared control animals by multiple researchers

- Archiving of frozen tissue samples to permit analyses of novel factors without additional in vivo experiments. We are creating a tissue bank of 10 different tissues from experiments using mice lacking all microbes or with specific microbes.

- Archiving of strains not in current use by freezing embryos that can be thawed to reanimate the strain at a later date if needed.

- Where possible both genders will be equally used across experiments.

- 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.

- To reduce the possibility of genetic drift due to inbreeding, we will backcross genetically modified strains to the parent strain approximately every 10 generations 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 are using a model of cystic fibrosis with a genetic mutation in the lung that is corrected in the gut by the expression of the human version of the gene. This prevents gut symptoms that would otherwise cause death within the first few weeks after birth. In contrast, the gut corrected model we will use shows no gut symptoms and few overt lung symptoms. Other genetic models may cause an increased susceptibility to rectal prolapse, but this may be reduced in a clean facility and by the absence of microorganisms. We will keep our strains in different microbial environments, including the total absence of microorganisms and with known microorganisms. Where possible when breeding mice with different microbial status we will alter the microbes of the mother before mating, so that the pups are born with the correct microbes without needing to perform direct intervention on them.

We will give non-harmful and pathogenic bacterial and fungal species to mice with and without the genetic alterations described. The microorganisms will be given orally to infect the gut, or through the nose or directly into the lungs to infect the lungs. Where pathogenic species are given we will monitor animals daily and terminate experiments typically within 2 weeks following infection. We may keep animals longer (up to 6 months) with non-pathogenic microorganisms to assess the longer term impacts on the immune system, but

as these bacteria are not disease-causing and might be found in healthy animals and humans we don't expect this to cause any adverse effects.

Why can’t you use animals that are less sentient?

The mouse is the most appropriate species for our studies because the mouse models that we will utilise are the best characterised models of cystic fibrosis and the other genetically-driven mucosal inflammatory diseases we are modelling, and re- capitulate the main features of disease. The mouse is the lowest neurological sensitive species that shows similar intestinal and lung disease to the human, and for the diseases that we are researching (cystic fibrosis in particular) we need a fully functional mammalian immune system which is not possible in fish or flies.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

To investigate the role of pathogenic and non-pathogenic microorganisms it is necessary to allow inflammation to develop to a moderate degree and the different disease characteristics mean that the

end points will differ in different models. We are using the most refined models of the diseases we are modelling, as they minimise the clinical signs for the animal whilst recreating many of the molecular processes involved in disease progression.

We have established refined monitoring schemes tailored to different procedures to assess the extent of clinical signs of gut and lung inflammation, and to detect any adverse effects in experimental animals. We will shorten our models to reduce suffering to the shortest possible time, and our monitoring schemes allow us to end experiments as soon as practically possible after disease has developed.

We use environmental enrichment and reduce social isolation of mice to a minimum by co-housing wherever possible. We make every effort to keep the number of procedures each animal undergoes to a minimum, for example by combining injections wherever possible. We also carefully consider the route of injection and where multiple routes are possible we use the least painful and invasive.

When possible and necessary, we use anaesthetics to reduce temporary discomfort during a procedure. Our preferred choice of anaesthetic is inhalation, which we use whenever possible, although it may be less appropriate when performing lung infections. The equipment we use is very reliable to ensure the appropriate level of anaesthetic is delivered.

When using live pathogens, we use species that primarily infect the mucosal surfaces, and avoid the use of species and routes that cause more systemic disease.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We make use of the ARRIVE, LASA and PREPARE guidelines both for planning and reporting experiments.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will regularly attend animal welfare meetings. When utilising new treatments or models we will seek the advice of experts in the field to ensure that we are using the most appropriate techniques and monitoring. We use the NC3Rs website to keep up to date on more general advances in the 3Rs (https://www.nc3rs.org.uk), and have access to a regional NC3Rs manager. We will also discuss 3Rs with the local Named Information Officer.

56. Zebrafish models to investigate disease processes associated with brain haemorrhage

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Zebrafish, brain haemorrhage, blood vessels of the brain, disease biology, drug discovery

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 identify and investigate molecules that can regulate the stability of the blood vessels of the brain and/or regulate brain injury in models of human diseases associated with bleeding in the brain, such as stroke.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Brain bleeds, also known as 'brain haemorrhages', are a type of stroke that account for almost 6% of all global deaths. We do not fully understand the biological mechanisms that

cause blood vessel weakness that lead to brain haemorrhage and we have no specific medicines that target the brain injury for patients once the brain haemorrhage has occurred. This project will allow us to continue to investigate the disease mechanisms and also identify and test potential drug candidates for the treatment of diseases associated with brain haemorrhage.

What outputs do you think you will see at the end of this project?

We will generate new data that will expand our understanding of the disease biology associated with brain haemorrhages and related cerebrovascular diseases. Our work will identify new molecules that may be developed into candidate compounds for progression to clinical trials. We will also continue to produce a number of publications that describe our work. We will also present our data at national and international scientific conferences. In this project, we will engineer some new zebrafish with different genetic backgrounds which we might share with other researchers. The outputs from this project will allow us to continue to provide evidence that zebrafish disease modelling is a suitable alternative approach for pre-clinical brain haemorrhage research that can reduce the numbers of mammals required for these types of studies.

Who or what will benefit from these outputs, and how?

The immediate impact of these outputs will be for our research group. Longer term these outputs may benefit both the wider pre-clinical and clinical stroke research communities and zebrafish researchers. Based on subsequent development of candidate compounds identified in this project, patients and the pharmaceutical industry may potentially benefit from our outputs. More broadly, animal welfare may also benefit, as we aim to reduce the numbers of mammals required for this type of research.

How will you look to maximise the outputs of this work?

We will regularly publish our work in open access scientific journals and present our data at national and international scientific conferences. As we have done previously, all of our datasets will be deposited in open access online resources. We will continue to collaborate with researchers and funders in the UK and internationally and share our knowledge/material with them. Wherever possible negative results will be included in publications (e.g. through supplementary appendices) or deposited through online resources.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 46,500

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.

In terms of Home Office regulations, zebrafish are animals with the lowest neurological complexity that can be genetically modified to study human diseases associated with brain haemorrhage. Our previous project licence has allowed us to establish zebrafish as an

excellent model for cerebrovascular disease research and has indicated that they could be used more widely to reduce the numbers of rodents required for this type of research. Importantly, zebrafish embryos, larvae and juveniles allow for live imaging and microscopy protocols to observe cellular responses throughout the entire depth of the brain in living animals - a technique that is currently not possible in mammalian models. Due to their small size and abundant numbers, zebrafish embryos are also required for drug screening protocols, where thousands of drug compounds can be tested in a relatively short period of time - which is not feasible in such a short time frame in mammals. Genetic modification is also very well established in zebrafish and more efficient than in mammals. Therefore, we can easily generate colonies of genetically modified adult zebrafish that can be bred regularly to generate embryos/larvae/juveniles for experiments.

Typically, what will be done to an animal used in your project?

Different procedures will be performed on animals at different life stages.

Genetically modified adult zebrafish will be generated through manual microinjection of nucleic acids into fertilised embryos and raised to adulthood in the aquatics facility. To confirm genetic modification, some adult animals will receive brief general anaesthesia so that a tail fin biopsy can be taken for genetic analysis or held in a fish net so that a surface skin swab can be taken for genetic analysis.

To note, zebrafish aged up to 5dpf are not protected under the Animals Welfare Act - and during these ages are commonly referred to as 'pre-protected'. For most experiments, zebrafish that display brain haemorrhage during the pre-protected stages will be used. Brain haemorrhage will be induced at 2- 3dpf either because of a genetic defect or through exposure to a chemical (e.g. atorvastatin). Both approaches causes the blood vessels in the brain to burst at only this very early age. We will use these animals to observe how the brain injury caused by the haemorrhage resolves over time.

In some cases, experimental animals older than 5dpf will be used to study brain injury through live imaging using microscopes, and some of these animals will be immobilised in low-melting agarose. If necessary (based on age of fish), immobilised fish will be placed in an imaging system that contains a water flow to maintain oxygen supply for the animals. During this course, chemicals may be introduced into the medium surrounding the fish.

In some cases, swimming behaviour will be recorded in animals. This will either be done in a tissue culture dish or in an observation tank. Animals will be briefly anaesthetised prior to assignment of experimental groups.

In some cases, zebrafish will be bathed in water containing a chemical/drug. Alternatively, zebrafish will receive a specified dose of chemical/drug by intraperitoneal, intravenous, intramuscular or retro-orbital injection, possibly repeated over several cycles, under terminal anaesthesia. In some cases, terminally anaesthetised zebrafish will receive an intravenous injection of a fluorescent tracer dye and prepared for live imaging. To note, the chemicals/drugs used will be chosen based on either their 1) potential to improve brain injury outcomes or 2) potential to weaken blood vessels of the brain.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals that are recovering from anaesthesia during the project might display abnormal swimming behaviour. If this behaviour persists for 30minutes after the removal of anaesthetic, then such animals will be humanely killed using a schedule 1 technique.

Any fish exhibiting abnormal behaviour or signs of infection following genotyping (where animals are briefly anaesthetised so that a small piece of tail fin can be dissected for DNA analysis) will be humanely killed by a Schedule 1 method. For most zebrafish tanks, this will occur in less than 2% of fish.

Some fish may have the potential to develop harmful neurological observable traits after a certain age, which would appear as abnormal swimming behaviour. In all cases, these animals will be humanely killed before reaching that age and before these signs start, unless moved on to another protocol as continued use for a specific purpose.

Fish exhibiting any unexpected harmful observable traits will be humanely killed, or in the case of individual fish of particular scientific interest, advice will be sought promptly from a Home Office Inspector.

Fish that display signs of suffering that affects their health and wellbeing such as abnormal swimming, slow growth or abnormal feeding will be immediately humanely killed.

Some anaesthetised animals that undergo injections may suffer from bleeding, but any pain will be controlled by analgesia as advised by the NVS.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

For obtaining sperm/eggs and breeding and maintenance of zebrafish = 80% mild, 20% sub-threshold. (NB: Sub-threshold is a severity level where no pain or suffering is observed).

For experiments to assess progression/recovery from brain injury in protected zebrafish = 100% moderate

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?

Although experiments using cells or in test-tubes can be informative, these systems cannot mimic the complex interactions that occur between blood, blood vessels, brain cells

and the immune system in conditions associated with brain haemorrhages. The only way we can perform the necessary experiments to understand the biological processes that occur in the brain is to use animal models– where the natural environment within the brain remains intact.

Historically rodents have been used to study haemorrhagic stroke. Zebrafish are animals with lower neurological complexity than mice and rats. As such, the use of zebrafish embryos and early-stage larvae can be considered as a partial replacement of the existing rodent models. Furthermore, many of the experiments performed in this proposal will be performed on fish embryos and larvae during the pre-regulated stages, which we know experience minimal distress and recover quickly after brain haemorrhage.

Which non-animal alternatives did you consider for use in this project?

We consider and do regularly perform some experiments in cell culture models (e.g. human brain blood vessel cells) - to help verify discoveries made in the fish model. We can also use brain tissue obtained from people who died from brain haemorrhage in a similar way. To aid with drug development,

we can use computer modelling to help understand how a drug might interact with a particular biological target. We can also consider the use of existing clinical trial data to help support the relevance of any potential new treatments we make in the fish model.

Why were they not suitable?

We try to incorporate non-animal experiments into our research wherever possible. Although experiments using non-animal approaches can be hugely informative, these systems cannot mimic the complex interactions that occur between blood, blood vessels, brain cells and the immune system in conditions associated with brain haemorrhages. Furthermore, for drug development, candidate compounds must be first tested in animal models before consideration for human 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 numbers of animals we predict to use is based upon feedback from our researchers of the numbers of larvae they predict to use and the number of parents we would require to breed to create the required number of larvae.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

For specific experiments we will continue to work with statisticians to ensure our sample size calculations remain accurate. Wherever possible, we will use fish that are siblings as our control comparison groups. For example, we will be able to generate young fish that

carry zero, one or two copies of a genetic defect from breeding parent fish. As these young fish are brothers and sisters, it means that we can make better comparisons between these animals for our experiments, whilst also reducing the need to produce additional young fish from other parent fish.

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 each experiment we will collect tissue wherever possible to share with other researchers. We will bank tissue from surplus embryos and store in the freezer for future pilot and optimisation studies. Researchers will share embryos from each breeding pair/tank for experiments to avoid unnecessary overproduction of embryos. However, regular breeding of adults is essential to ensure longevity and to avoid health problems (e.g. egg bound females).

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Most of the work in this project will involve the use of pre-protected zebrafish larval models that exhibit brain haemorrhage between 2 and 3 dpf. We have shown that these larvae recover by the age of 5dpf. Therefore, minimal welfare issues are expected with this model.

Some of these animals will be grown beyond 5dpf into the regulated stages and assessed to better characterise the biology associated with the recovery process after brain haemorrhage. As these animals are recovering, we do not expect any significant welfare issues, but will monitor them closely to ensure no signs of distress or suffering are apparent.

Some of these animals will be grown beyond 5dpf into the regulated stages and assessed to determine if there are any weaknesses in the blood vessels of the brain. This may involve injection of drugs and/or fluorescent dyes into animals. These fish will be anaesthetised to control pain from injection prior to imaging. The animals will be killed immediately after the experiment to avoid lasting harm.

Why can’t you use animals that are less sentient?

Zebrafish are the least sentient vertebrate species available that can be used for studying haemorrhagic stroke and related conditions. Wherever possible we will use pre-regulated embryos and larvae for our experiments. A large component of our project will involve live imaging in animals under terminal anaesthesia.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

As the project progresses, phenotypes that are observed that are deemed to be associated with cerebrovascular disease will be recorded to allow us to categorise the clinical signs based on severity banding, to allow us to generate a future 'traffic light' system, as implemented for rodent models of stroke.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will closely follow updates on practical guidance for zebrafish research published by the NC3Rs. We will also attend international Zebrafish conferences on an annual basis to receive the most up to

date published best practice guidelines for experimental procedures. We will continue to design and complete experiments in align with the ARRIVE and PREPARE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our research has received past and current 3Rs-related funding and we have regular contact with our in-house 3Rs team. As such, we are regularly informed of the latest advances in the field with regards to the 3Rs and the use of zebrafish in scientific research. Wherever possible we will implement such advances in our protocols, for example as we have done for zebrafish skin swabbing as a refined protocol for genotyping. We also have internal 3Rs-based seminars and workshops, and good communication with our animal unit and other zebrafish lab users that we can discuss best practice with.

57. Immune control of fungi in health and disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Fungi, Allergy, Immunity, Inflammation, Metabolism

Animal types	Life stages
Mice	juvenile, adult, pregnant, embryo, neonate

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to understand how fungi cause the immune system to drive inflammatory disease. By extension, we aim to learn how these processes are regulated by the local tissue environment and how when this is disrupted it can lead to worse anti-fungal diseases such as as severe asthma with fungal sensitisation.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Fungi are abundant in the environment (for example thousands of spores are breathed in every day), and are able to colonise a range of barrier mucosal tissues in the human body (e.g. lung, intestine and skin). Our immune response has developed strategies to respond and prevent opportunistic fungi invading host tissue. However, constant exposure to fungi can lead to local inflammation which may trigger the development of severe chronic

inflammatory diseases like asthma and chronic tissue scarring (fibrosis). Furthermore, these anti-fungal chronic inflammatory responses are known to be a major factor in worsening chronic airway diseases (such as cystic fibrosis) and metabolic disorder disease (such as diabetes and obesity). Despite the huge burden on global health (there are 300 million asthma sufferers worldwide), the precise events and signals that lead to immune cells triggering inappropriate responses against fungi leading to chronic disease are poorly understood. Thus, fundamental research into the mechanisms and consequences of the anti-fungal inflammatory response will lead not only to improved understanding of fungal-host interactions, but greater understanding of the many chronic inflammatory diseases and should also provide proof of principle data for future therapeutic development.

What outputs do you think you will see at the end of this project?

A central goal of this project is to identify new understanding of the mechanisms that lead to fungi triggering immune cells to initiate, enhance or maintain inflammation that underpins several chronic diseases.

We have identified important immune cells that are involved in mediating chronic anti-fungal inflammation. We aim to generate vital data to help understand how the precise immune and fungal signals that lead to these responses. Furthermore, as part of this project we also want to address how underlying chronic conditions (such as cystic fibrosis and metabolic disorders like obesity and diabetes) change these processes by altering the tissue environment of the lung leading to enhanced anti-fungal inflammation worsening disease outcomes.

This project aims to disseminate new knowledge by publication in peer reviewed journals and presentations at conferences, seminars and workshops. We hope that in the longer-term our work will contribute to new immunology- and anti-fungal based therapies for chronic inflammatory diseases. In the 5 years of this project, we aim to continue our high standard of publication, averaging more than 5 research papers per year in highly-respected peer-reviewed journals.

Who or what will benefit from these outputs, and how?

This project aims to answer fundamental scientific questions. The new knowledge generated, and the unravelling of important immune mechanisms, will be relevant to a broad range of human and animal conditions.

In the short term (over the time period of this 5-year project), we will gain a new understanding of how cells of the immune system are triggered to elicit chronic inflammation against fungi that are abundant in our environment. Importantly, we will also screen current therapies (e.g. anti-fungal drugs) and understand whether they would be suitable to treat these responses in our animal models. Within this project we will also translate these findings utilising samples collected from patients with chronic inflammatory disease (such as asthma) to confirm which features are reflective of human disease.

In the longer term, beyond this 5-year project, we aim to develop novel therapeutic candidates based on our fundamental research into chronic anti-fungal immune responses. These novel candidates could be targeted individually, or combined with established therapeutics, to improve current treatment strategies in a range of inflammatory diseases.

How will you look to maximise the outputs of this work?

Communication of our findings will be primarily through publication in widely-read peer-reviewed journals, but also presentation at local, national and international congresses and institute seminars. To ensure maximum dissemination, only journals that allow open access without payment by the reader will be considered. To prevent unnecessary repetition of experiments by others, we will seek to publish all data generated under this project, including negative results.

To enable rapid translation of our findings to the clinic we will exploit new and existing collaborations, and with local clinicians as part of the translational environment within our institution. We have highly effective systems in place for technology transfer. Additionally, we aim to expand our current collaborations with pharmaceutical and biotech companies by presentation at national and international forums at which industrial representatives are present.

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.

We study adult mice because the immune system, tissue organisation and development of all mammals are similar, allowing mice to be a model for humans and other animals. Mice are also used because scientists have created many genetically altered mouse lines that allow us to dissect in fine detail what happens during immune responses in response to fungi. Genetically altered mice, and many of the tools designed to work with mice, allow us to define in precise detail how particular cells and molecules of the immune system work together to fight fungal infection. By manipulating these cells and molecules, we can identify the immune components that co-ordinate anti-fungal responses that potentially lead to chronic inflammatory disease, and use that information to help design future therapies.

Typically, what will be done to an animal used in your project?

Typically, animals will receive repeat doses of fungal spores (and/or other allergens) to mediate chronic anti-fungal inflammation. In many cases mice will also receive a single or multiple injections containing an immunomodulatory substance (e.g. antibodies to neutralise a specific immune mediator or deplete a specific cell type, or cells to promote a particular response) (Protocols 2 and 3). In some instances mice that have sustained energy imbalance, typically via high-calorie feeding or using genetically altered mice (e.g. deficient in specific genes of interest) aimed at disrupting the biological process of energy balance (also called energy homeostatsis, i.e. the co-ordination between food intake versus energy expenditure), will be repeatedly dosed with fungal spores and/or other allergens followed with single or multiple injections containing an substance that disrupts the immune response (Protocol 4).

Experiments using Protocol 2 might look at the immediate immune response in the first few days after administration of fungi or immunomodulatory substance, or may last up to 8 weeks with repeat dosing (up to 5 doses a week, primarily through intranasal administration) to allow assessment of chronic anti- fungal immune responses. In addition to spores, mice might receive a single or multiple doses of an immunomodulatory substance (e.g. once a week for 2-3 weeks).

Mice on Protocol 3 will receive cells (either pulsed with or without spores) either prior to, or after, treatment with a substance that disrupts the immune response. These might then be further challenged with fungal spores and/or allergens. For Protocol 2 and 3, these experiments will typically last between 1 and 20 days, though longer experiments may last up to 8 weeks).

Mice that have an altered energy balance (Protocol 4) will be assessed for altered energy activity (testing metabolic function and glucose handling) in models of altered energy-sensing (e.g. transgenic or diet-induced obese (DIO)). These mice will then be dosed repeatedly with spores (and/or allergens) to mediate chronic anti-fungal allergic inflammation. In addition to, or instead of, mice might receive a single or multiple doses of a substance that disrupts the immune response (e.g. once a week for 2-3 weeks). These experiments may last up to 30 weeks.

The cumulative experience of mice will typically be exposure to 3 or 4 procedures (if repeat intranasal dosing is regarded as a single procedure) that may each cause short but usually separated periods of typically mild or potentially moderate degrees of suffering.

Experiments will end with animals being killed humanely. For the majority of experiments this will performed by exposing mice to rising carbon dioxide levels. This schedule 1 approved approach is the best approach for our research as it enables us to isolate samples from the airway. Other methods such as cervical dislocation cause damage to the trachea) preventing us taking airway samples. In rare instances (approximately 5%) we need to euthanise the animal under terminal anaesthesia due to potential damage to lung tissue by being exposed to high levels of carbon dioxide (e.g. when studying epithelial cells that line the lung airway).

What are the expected impacts and/or adverse effects for the animals during your project?

The vast majority of animals will experience no adverse effects or only mild adverse effects.

Our models of repeated fungal spore (or allergen) are generally very well tolerated, though in rare cases can cause weight loss and affects balance (e.g. head tilting). Furthermore, in very rare instances (especially if allergens are given over a long time period) breathing problems, i.e. laboured breathing, increased/decreased respiration However, in all cases these will rarely reach moderate severity.

Although immune modulation can trigger systemic inflammation that can cause weight loss, raised hairs (piloerection), hunching and reduced movement, in most cases these effects should be mild or transient.
In some instances, animals will gain excess body weight (primarily fat mass) and begin to display symptoms of impaired glucose handling and insulin resistance following prolonged

periods of positive energy balance (DIO). Symptoms may include excessive urination (polyurea) and reduced physical activity. Animal husbandry will be adjusted to maintain a comfortable housing environment for these animals (remove and replace wet bedding) and body condition scores will be utilised to monitor animal welfare.

In all experiments, animals will be carefully monitored and humanely killed before they exceed moderate severity limits. Guidance will be sought from the NVS and NACWO should any animal display signs of abnormal behaviour or any unexpected change in physical appearance.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We expect approximately 85% of mice to experience mild severity and 15% of mice to experience moderate severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The mammalian immune system is highly complex, relying on the co-ordinated actions of multiple different cell types and molecules that collectively provide protection. As such, the insight in vivo experiments can provide on the mechanisms that underpin anti-fungal chronic inflammation is of significant relevance. Importantly, we know the importance of the lung microenvironment and its ability to govern immune responses. Therefore, in vivo models allow us to manipulate this environment and understand how chronic underlying disease (e.g. like obesity and diabetes) alters anti-fungal immunity.

Unfortunately, in vitro systems are unable to reflect the cellular and molecular complexity of the immune system and lung barrier microenvironment. Therefore, the use of mammals is essential for gaining a better understanding of the mechanisms underlying immune protection that could be utilised for patient benefit.

Mice will be used in these studies because their immune system closely resembles the human immune system therefore giving a better chance for translating potential therapies. Additionally, a wide array of wild type and genetically altered strains of mice are available that will allow us to better decipher the role of immune cells and molecules in anti-fungal immunity. Finally, a vast range of reagents is available for analysing mouse cellular and molecular interactions during immune responses.

Which non-animal alternatives did you consider for use in this project?

1) Analysis of samples collected from the human airway.

2) Use of cell lines and in vitro systems (e.g. organs on a chip platform).

Where possible, we will use in vitro assays to provide initial data on the effects of immunomodulatory substances on specific immune cells. This data will then be used to inform and complement our in vivo experiments.

Why were they not suitable?

The types of experiments required to track cell function in vivo are not possible with human tissue biopsies, nor can we experimentally manipulate humans.

Many location-specific features of cells are lost once they are removed from the tissue, which makes the use of cell lines impractical. In vitro systems typically allow for the study of one or two cell types in a highly controlled environment that is not reflective of the complex immune system in vivo. Therefore, to fully understand how different cell types and molecules co-ordinate an effective immune response we require in vivo experiments.

We will regularly review the literature regarding in vitro systems (such as recent developments in organs on a chip technology which can recreate aspects of tissue physiology with cultures of cells) so that if new approaches are developed, we can test them and potentially exploit them if they succeed.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of mice has been estimated based on extensive experience gained during my career working on several Home Office licenses, taking into account breeding strategies for genetically altered mice, and anticipated numbers of planned studies over the course of the license.

For the length of this project we expect to use 5500 transgenic mice and 3500 wild type mice (9000 in total). This level of activity is inline with our current usage and future projections for the research that will be undertaken by my group.

We anticipate that approximately 5500 mice will be bred and maintained from transgenic mouse colonies. These will be managed across 2 breeding protocols (5000 mice in protocol 1, 500 mice in protocol 5). Of these we anticipate 3500 will be transferred to experimental protocols.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

For all of our experiments, in-bred mice are used to reduce experimental variation, which makes it possible to use fewer animals to achieve statistical significance. For the majority of our studies, mice from the same litters are used for control and experimental mice, reducing variation that can occur due to differences in the microbiota. Overall, our experiments are designed to reduce the number of variables (for example age) to as few as possible and thereby reduce the number of control groups required.

We work with the NC3Rs Regional Programme Manger to ensure all lab members are introduced to the NC3Rs experimental design assistant and encouraged to use it. Everyone in the lab is trained in statistical methods and these are regularly discussed at lab meeting, to ensure all agree the best methods are being used. This includes randomisation and blinding, whenever practically possible.
Tissue-sharing is a major tool we use to reduce animal usage.

A significant proportion of our animal use is related to breeding programmes for genetically altered lines. We follow the advice of our animal facility staff to optimise breeding, and regularly discuss numbers at lab meeting to ensure we do not overbreed. Where possible and appropriate, we use antibody blockade of wild type mice, instead of gene deficient mice.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We routinely perform pilot experiments to determine the optimal number of mice to achieve statistical power. Experiments are then performed on a minimum of two separate occasions to ensure reproducibility, following which data pooled from experiments are statistically analysed to reveal less pronounced effects without increasing overall animal use.

We have many years of experience in planning animal experiments and we plan our research to ensure that all animals are used most effectively. We often combine experiments to ensure that multiple organs are used to address multiple objectives at once. Careful discussion between multiple researchers is required to avoid compromises. Due to high variability in immunological and models in vivo, especially in genetically altered models that we have yet to assess, we will adjust group sizes as required, should subsequent power calculations indicate that this is necessary.

The increased use of genetically altered animals has led to more complicated breeding strategies and, as a result, larger colonies. We reduce the numbers of these animals in our experiments by using littermates as controls wherever possible. Additionally, when a particular strain is not being used experimentally, we work closely with the animal technicians to develop a breeding strategy that maintains low numbers of stock animals.

In many experiments, we will use bone marrow to carry out pilot studies in vitro, therefore only a few animals are used to initially test new hypotheses. Importantly, we continue to work closely with collaborators who can supply bone marrow samples, therefore reducing the number of genetically altered animals that have to be bred to facilitate experiments.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the

procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use mouse models to study how constant exposure to fungi causes the immune response to mediate chronic inflammation at mucosal barrier sites. Mice represent the most appropriate species for in vivo study of anti-fungal immunity, because of the extensive knowledge of their physiology as it relates to humans, the genetic and biological tools available and the ability to be easily bred and handled.

The chronic inflammatory models we will use do not cause significant pathology, and doses and timing are carefully managed such that the animals will experience minimal suffering. As our experience of these models develops, we will look to refine our approaches to ensure robust experimental results whilst minimising pain, suffering or distress.

Another possibility to reduce and refine mouse usage is the utilisation of zebrafish models. Whilst the zebrafish model has progressed in terms of modelling fungal infections, there are still significant

limitations for its use in understanding immune complexity in immunology research. In particular, there are significantly fewer validated immunological tools and reagents available for zebrafish than for mice, reducing the depth and breadth of cellular and molecular understanding that is achievable. We will regularly review the literature regarding zebrafish systems so that if new approaches are developed, we can plan to exploit them.

Why can’t you use animals that are less sentient?

To our knowledge, no other species of lesser sentience can fulfil the requirements of this project to the same extent as the laboratory mouse. We are studying long and complex immune processes, and trying to understand how the adult immune system handles immune responses against factors that mediate anti-fungal inflammation, and how different cells communicate to orchestrate an appropriate response. Only adult animals would give meaningful results. To define the impact of immunomodulation and metabolic disruption on anti-fungal immunity requires mice to be monitored for several weeks, so procedures cannot be carried out on terminally anaesthetised mice

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All procedures will be performed by trained and skilled personal licence holders, who will handle animals with care. Animals will be monitored for adverse effects using score sheets previously developed in conjunction with the NVS and NACWO. These score sheets have proven to allow for objective measurements of clinical signs associated with adverse effects to determine when humane endpoints have been reached.

In line with the establishment's policy, we will adopt the latest techniques in animal handling (e.g. tunnel handling) to significantly reduce the stress associated with procedures. Furthermore, where possible, the least invasive methods for dosing and sampling will be applied.

Anaesthesia and analgesia protocols will be refined, through discussion with the NVS, to ensure they are the most appropriate for each type of procedure.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow LASA guidelines, and consult the recommended https://www.nc3rs.org.uk/3rs-resources on a regular basis, including watching videos of best practice techniques. For specific models, we read papers from other groups doing similar experiments, as well as consulting directly with other researchers to discuss the most refined procedures.

We will continue to use the NC3Rs online tools. This will include use of the the Experimental Design Assistant to ensure we design experiments that will allow us to achieve statistical significance whilst minimising the number of animals we need to use. We will also utilise the 3Rs self-assessment tool to ensure my research group actively discuss 3Rs and how we can continually refine and improve our practice.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will hold frequent discussions with NACWOs, NIO and NTCO within our animal facility along with a team of dedicated veterinarians seeking to continually improve animal welfare and refine animal use. These are further facilitated by regular open meetings with NAWCOs, NIO, NTCO and all personal license holders participate to ensure we are following current best practices. We will also continually keep track of the latest advances in improving animal welfare via discussions with colleagues, attending national/international conferences and consulting published literature. We will continue to work closely with our local NC3Rs representative, and consult the NC3R website to ensure we stay informed about the advances in the 3Rs. For example, we utilise the NC3Rs Experimental Design Assistant website when planning experiments to ensure we will achieve our scientific aims with the minimal number of mice.

58. The cell biology of zebrafish infection

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Bacteria, Host defence, Infection, Zebrafish

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?

We will study host and pathogen determinants underlying bacterial infection of zebrafish.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 zebrafish (Danio rerio) has emerged in the last two decades as a powerful non-mammalian vertebrate model to study the development and function of the immune system. It is a genetically modifiable organism, sharing immune pathways and cells with mammals. The small size and natural translucency of zebrafish larvae make it possible to follow individual cells and their behaviour in vivo throughout the organism, in a totally non-invasive manner. We previously established conditions for experimental infection of zebrafish larvae that allow real-time imaging of bacteria-leukocyte interactions at high resolution. We herein propose to exploit the transparent nature of the zebrafish larvae and straightforward genetic manipulation to study host and pathogen determinants underlying bacterial infection in vivo. This information should provide vital clues towards understanding bacterial disease and for illuminating new therapeutic strategies.

What outputs do you think you will see at the end of this project?

A major aim of this project is to provide insights into mechanisms required for the control of infection and will expand the potential of our infection model to study clinically relevant science. The results generated by the end of this project are expected to improve significantly our understanding of pathogenesis and the cell biology of infection, and will be submitted for publication to peer-reviewed journals of international standing.

Central to this work is the ability to investigate bacteria-host cell interactions using live imaging in a transparent zebrafish model, which allows us to study (non-invasively) the progression of infection in real time for both fundamental and translational impact. The data generated is both qualitative and quantitative, and will benefit the greater infection biology community (including researchers working in the fields of microbiology, immunology and cell biology). The work can also have direct clinical relevance in that it will innovate therapeutic strategies to combat antimicrobial resistant infections in patients.

Who or what will benefit from these outputs, and how?

The short-term benefits include presenting our findings at scientific conferences and publishing discoveries in internationally recognised scientific journals. Medium- and long-term benefits include generating a deep understanding of the zebrafish immune response to infection, fostering international collaborative ties, and improving therapeutic treatments to bacterial infections.

How will you look to maximise the outputs of this work?

This research programme will generate data outputs of varying size and complexity. For example, lab and electronic notebooks, presentations and scientific posters will be produced.

All data will be stored securely. Raw electronic data will be stored with metadata to ensure curation and retrieval, with accurate notations in lab notebooks reflecting file names and persistent locations. Raw data and detailed methods (including analysis software) underpinning open access research articles will be made available to other researchers at the time of publication through supplemental figures or data sharing repositories. All research publications will be available as preprint (eg bioRxiv) and publication will be in accordance with policies on open science.

This research programme may generate intellectual property in the form of data, results and materials. This may include patent protection and involve third party collaboration.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 15250

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 host response to infectious disease is a complex trait, influenced by multiple factors, including virulence of the pathogen and the host genetic make-up. Teasing out the multiple factors that contribute to infection in humans is extremely challenging as many of the variables remain unknown. Using animal models to study infection, we can focus on individual factors during the infection process to establish their relative importance. In this way, animal work proposed for this research programme should provide vital clues towards understanding human infectious disease, and possibly other human diseases arising from a dysfunctional host immune response.

The zebrafish is a powerful vertebrate model to study the development and function of the immune system. We can modify its genome using well established techniques (eg CRISPR), and the zebrafish immune system shares striking homologies with the mammalian immune system. Moreover, the small size and natural translucency of zebrafish larvae make it possible to follow individual cells and their behaviour in vivo throughout the animal in a non-invasive manner.

The majority of experimental bacterial infections will be performed in larvae up to 5 days post fertilisation (dpf) (i.e. they are not protected animals under The Animals (Scientific Procedures) Act 1986). However, some work will be done at the juvenile and adult stages. This will depend on the pathogen under study (i.e. acute pro-inflammatory infection vs chronic infection) and the development of adaptive immunity in zebrafish (which is fully mature and functional from ~28 dpf). The use of any new infectious agent in zebrafish infection studies will be carefully evaluated by carrying out pilot studies, and the work will be fully discussed with the AWERB committee.

Typically, what will be done to an animal used in your project?

Zebrafish colonies will be generated through breeding or genetic manipulation of gametes.

The majority of experimental work with infectious agents or their derivatives will be performed in larvae up to 5 dpf. However, to study infection establishment, progression and development of chronic infections, we may need to follow infection in zebrafish > 5 dpf. We will also study the importance of adaptive immune responses, and therefore juvenile or adult fish may be injected with infectious agents or their derivatives. To understand the development of antimicrobial resistance, we will also add antibiotics to the zebrafish housing water.

Infected animals will be closely and frequently monitored, and when adverse effects are observed, they will be humanely culled by schedule 1 method.

What are the expected impacts and/or adverse effects for the animals during your project?

Expected adverse effects can include abnormal swimming behaviour, separation from the shoal, abnormal scale position, abdominal swelling, and abnormal skin outgrowth.

Infection may cause physical or behavioural abnormalities such as abnormal swimming posture/motion (at an angle/swimming in circles/bobbing vertically), dropsy (scales puff outwards), surface lumps which are large/ulcerated, surface lesions or wounds, rapid gill movement/gasping and lethargy. These symptoms may occur 2 days post-infection.

If zebrafish exhibit any of these adverse effects they will be culled by Schedule 1 method.

Animals will be frequently monitored, on a daily basis, so that any abnormality can be rapidly assessed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Adult zebrafish will mostly be used for breeding (80%, severity: mild) and a small fraction will be used for infection studies (20%, severity: moderate).

Animals will be closely monitored, on a daily basis, and will be humanly killed if they show signs of health deterioration or when the study has ended.

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?

The host response to infectious disease is a complex trait, influenced by multiple factors, including virulence of the pathogen and the host genetic make-up. Teasing out the multiple factors that contribute to infection in humans is extremely complex as many of the variables remain unknown. Using animal models to study infection, the researcher can focus on individual variables during the infection process to establish their relative importance. Animal work proposed for our research should provide vital clues towards understanding bacterial disease and for other human diseases arising from innate immune dysfunction.

Which non-animal alternatives did you consider for use in this project?

The use of replacement alternatives has been considered for this project and the majority of our research (90-95%) will be done before the embryos reach the independent feeding stage (<5 days post fertilisation; i.e. not protected under The Animals (Scientific Procedures) Act 1986).

Tissue culture models are routinely used by the laboratory to avoid animal models for examining the cell biology of infection in vitro. To validate these in vitro results, infections will be performed in zebrafish larvae to follow host-pathogen interactions in vivo (which cannot effectively be reproduced in the laboratory). Since our previous licence, we replaced a greater proportion of our work by in vitro models, reducing the number of animals used in this project.

Why were they not suitable?

Although in vitro models can illustrate important aspects of the infection biology of bacterial pathogens, we are also interested in dissecting complex interactions that occur between different cell types (e.g. macrophages and neutrophils), cross-talks between innate and adaptive responses (e.g. macrophages and B-cells), and the mechanisms underlying pathogen invasion and dissemination (e.g. brain infection that may spread to other tissues / organs). Therefore, we require a complex organism to investigate the multiple-layers of infection biology.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 animal numbers to be used in our future work is based on our previous license returns and scientific outputs.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We employed NC3Rs' experimental design guidance and experimental design assistant (EDA) to plan our experimental design, practical steps and statistical analysis using the advice and support for randomisation and blinding, sample size calculations and appropriate statistical analysis methods.

We will use the EDA diagram and report outputs to support experimental planning with animal users. Additionally, PREPARE and ARRIVE guidelines will be used to assist in the planning of experiments and in the reporting of animal experiments, respectively.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Most infection studies will use zebrafish larvae (<5 days post fertilisation), which enables replacement of experiments that would otherwise be performed with regulated animals such as mice and zebrafish. Infection experiments in adult zebrafish will be carefully planned, making use of statistical tools and seeking statistical advice to determine the minimum number of animals necessary for experiments.
Pilot studies will be carried out to define the minimum number of animals per group required to achieve appropriate statistical robustness.

Breeding strategies will be carried out by personnel with a PIL and aimed to avoid unnecessary animal generation (animal surplus).

Where possible, imaging at different timepoints will be carried out using the same zebrafish and tissues will be shared across experiments.

Experiments will be carefully planned to avoid duplications.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Zebrafish will be used because they are easily genetically modified, fast breeders and the larvae are small and transparent which make it possible to follow individual cells and their behaviour using a microscope. This is also an advantage as some transgenic lines can be genotyped using fluorescence imaging without involving invasive techniques.

The mouse model is not practical to visualise the subcellular roles of individual host factors during bacterial infection because of unsuitability for in vivo imaging at the cellular level. The zebrafish has emerged in the last two decades as a powerful non-mammalian vertebrate model to study the development and function of the immune system. In this research programme we are proposing to exploit the zebrafish model to study host and pathogen factors underlying bacterial infection in vivo. This should provide vital clues towards understanding bacterial disease, and potentially for other human diseases arising from innate immune dysfunction.

All breeding and husbandry procedures will be performed by trained staff, and the zebrafish monitored regularly. For environment enrichment, fish >21 days post fertilisation will be fed with live food.

As we will work with genetically modified animals, fish will be genotyped using available refined methods, and where possible, we will use fluorescence microscopy to screen for transgenic fish.

Colonies will be kept as homozygotes, where no adverse effects are found, to avoid having to genotype further generations.

To study adaptive immunity to bacterial infection we will work with zebrafish > 5 days post fertilisation. If required to study procedures in older animals, we will use a combination of analgesia with the anaesthetic. For any zebrafish displaying adverse effects there will be an increased monitoring schedule over the course of the experiment.

Why can’t you use animals that are less sentient?

Most infection studies will use zebrafish larvae (<5 days post fertilisation) which is suitable for investigating early innate immune responses. However, when investigating the longevity of innate immune responses or the impact of adaptive immune responses we require experiments to be done in adult zebrafish – as adaptive immune responses are only developed and matured from 4 weeks post fertilisation.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animal harms will be minimised by regular checking of the animals for relevant symptoms that constitute the endpoint of the experiment. All breeding and husbandry procedures will be performed by trained staff, and the zebrafish monitored regularly.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the RSPCA handbook on zebrafish husbandry, as well as NC3Rs, ZFIN and ZHA resources. All experimental procedures will be conducted following local best practice SOPs based on LASA guidelines and include the NC3Rs decision tree.

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 we have signed up to the NC3Rs newsletter. We will attend Regional 3Rs symposia. We will also have regular meetings with the NIO.

59. Control of blood flow in heath 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

Blood vessel, therapy, pharmacology, disease models of vascular disorders

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 is designed to uncover the mechanisms that control the function of small blood vessels in vital organs such as the brain and the heart, and to gain new insights into how alterations in these mechanisms may lead to debilitating diseases including stroke, vascular dementia and heart attack.

Much of our work focuses on the control of blood flow in the brain and how it is altered in diseases. We also aim to examine whether these mechanisms of control vary in various vital organs and how they can be controlled pharmacologically.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Alterations in the microvascular blood flow is involved in many severe human diseases (e.g. stroke, vascular dementia and heart attack). Understanding the mechanisms of regulation of small blood vessels is an essential step for the development of new therapies for the prevention and cure of these disorders. The work will reveal the specific drug targets that can be exploited pharmacologically for therapeutic benefit.

What outputs do you think you will see at the end of this project?

This work will lead us to identify key physiological mechanisms that control the function of small blood vessels within the vital organs of the body.

It will generate new publications within the scientific community and ultimately provide information that could lead to the development of new therapeutic drugs for diseases.

Who or what will benefit from these outputs, and how?

This project will have a variety of beneficiaries. In the short term, these will include the research communities involved in physiological studies of the cardiovascular system; they will benefit from the knowledge arising from the project, which is expected to foster additional key research around the world. In the mid term, the project has also the potential to translate into the development of new medicines for the control of small blood vessel function in debilitating diseases; in the longer term, this has the potential to benefit the patients and UK industry. The project will also benefit the undergraduate (medicine and biosciences) and postgraduate students in our institution and nationally, who will be exposed to the new advanced concepts in biomedicine produced during the project; this will contribute to their development as clinical practitioners and scientists.

How will you look to maximise the outputs of this work?

The output will be maximised in various ways. These include: (i) publications in peer-reviewed scientific and medical journals; (ii) conference presentations at major international meetings; (iii) established collaborations within the UK and abroad. The lab is also actively involved in public engagement of science and will make use of establishment’s webpages, live radio interviews, articles in funding bodies' newsletters, visits to schools and in general organisation of public engagement events to inform the public of the results of our research. We have significant experience in these kinds of activities and have organised several large-scale public engagement events in the past to show the general public how the heart, circulatory system, lungs, blood and kidneys work, and the therapies that can be developed to ameliorate vascular diseases of vital organs.

Species and numbers of animals expected to be used

- Mice: 5000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice have been chosen for this work as the lowest species which mimic human physiology and pathology well enough for our work to be relevant to human disease. In addition, mice are well characterised and widely used for the study of cardiovascular disease. Thus, their use will allow comparison and integration of our results with the existing literature.

We will primarily use mice of an age that corresponds to adulthood or mid-old age in humans, because many vascular diseases tend to occur after midlife in humans. In some experiments, younger mice may be used to study the effect of moderate ageing on some of the physiological processes that will be examined. We will also study the effect of gender on these processes. Therefore, mice of both sexes will be used in our experiments and any potential differences in effects observed will be statistically examined.

Typically, what will be done to an animal used in your project?

Animals will be bred and maintained in a suitable environment containing appropriate enrichments and controlled food and water provision. This is to ensure the optimal welfare of the animals maintained in the excellent facilities of our institution.

Animals will carry genetic modifications which may require induction (typically administered via oral gavage, but it may also be added to the drinking water) for the modifications to be activated. This procedure is considered “moderate”, because of the possibility of weight loss caused by tamoxifen; however, this does not occur in every animal.

Mice will be maintained typically until they reach adulthood (typically no more than 4 months), and then will be humanely killed.

What are the expected impacts and/or adverse effects for the animals during your project?

We do not expect our genetically altered mice to have a phenotype that is likely to cause any harms as the mutations are such that they make use of sensors of vascular cell function and are not expected to significantly interfere with the function of the cells. Other alterations are expected to cause some dilation of blood vessels which may have protective effects for problems such as stroke.

The administration of Tamoxifen to activate/deactivate genes may cause a transient weight loss of up to 15% for up to 5 days.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

50% Sub threshold, 35% Mild, 15% 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?

The project investigates the function of vascular cells within the blood vessel wall and in the tissues surrounding the blood vessels; experiments on isolated tissues cannot be replaced with cultured cells, because this would not allow reconstruction of the complex pattern of micro-anatomical and functional interactions that the vascular cells display with the adjacent cells. It is also not possible to reproduce the vast complexity of chemical and physical factors to which vascular cells are subjected to in vivo (e.g. blood pressure, exposure to circulating substances etc.). Thus, the use of animals (mice) is absolutely essential for this research.

Which non-animal alternatives did you consider for use in this project?

We have considered the possibility of using computer simulations as an alternative for our research. However, complete mathematical models of vascular function (including the effects of blood flow and blood pressure and chemical factors on the tone of these cells), and the impact of changes in small vessels tone (e.g. due to pericytes, contractile cells that surround capillaries) on the control of local blood flow in the microcirculation of vital organs such as the brain and heart, are not currently available. Part of our work has involved the use of live human tissue that is discarded from operations (neurosurgical, thoracic); thus, when possible these samples will be used for research. This approach, however, is not amenable to a large number of investigations with complex experimental design such as those planned for our research, meaning the use of animals (mice) is essential.

Why were they not suitable?

As noted in the sections above, complete mathematical models on microvascular function are not available and cell culture substitutes cannot fully replicate the complexity of the mammalian microcirculation. We can also perform some experiments on live human tissues discarded from operations. This approach, however, does not allow a large number of investigations with complex experimental design that is required for our in depth investigation. This implies that the use of animals (mice) is essential for our research.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers have been estimated using our previous experience and by the use of power calculations. These calculation have shown us that typically we require groups of 6-8 animals for each individual of set of experiment.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The NC3R's experimental design assistant has been utilised to devise an accurate experimental design in the context of appropriate animal usage. While our work requires animal experimentation, we have carefully implemented the 3Rs principles of Replacement, Refinement and Reduction.

In the context of Reduction, part of our work already involves the use of live human tissue that is discarded from neurosurgical operations. This approach, however, is not amenable to a large number of investigations with complex experimental design such as outlined above. Another consideration (outlined in greater detail in the previous section of this application), is that the project investigates the function of vascular cells within the blood vessel wall and in the perivascular tissues; experiments on isolated tissues cannot be replaced with cultured cells, because the complexity of these interactions cannot be fully reconstructed in vitro.

Nevertheless, some aspects of the research (including for example examination of the potency of pharmacological agents) will first be conducted in cultured cells. the results achieved from cultured cells will inform experimental design (e.g. optimal concentration of modulators to be used in ex vivo experiments) and reduce the number of animals needed.

As noted in a previous section of the application, computer simulations are not detailed enough to enable this research to be carried out via mathematical models. The data arising from this project, however, will help to define how the specific component of arterial cells (e.g. ion channels) control the function of small blood vessels (capillaries and arterioles) during a range of tissue and cellular challenges. The understanding of these mechanisms will help the generation of new, more realistic computer models of blood vessel function. This will have the potential to reduce, and to an extent replace, the use of animals in future research.

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.

We have published results involving isolated tissues, thus we have already quantified the variability of the parameters that we intend to measure as part of the current project; this knowledge was an essential requirement for performing accurate power calculations for the project.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use models of genetically altered mice with a mutation that affect component of cell in blood vessel that can control the diameter of the vessel and therefore the blood that can pass though the vessels.

Some of these models will require gene activation by the administration of a substance such as Tamoxifen. Typically, we administer this via the oral gavage which is one of the most refined routes of administration for these substances. We then allow the mice to mature up to ~4 months of age before we humanely kill them and harvest the tissues and organs of interest.

Why can’t you use animals that are less sentient?

The use of mice is essential for this research for these reasons: (i) mice appear to be one of the lowest species which mimic human physiology and pathology closely enough for our work on blood vessel function to enable extrapolation to humans; thus a mammalian system is required (ii) the genetic modifications we seek to use were generated in mice and not in other species; (iii) mice (including the strains used in the proposal) are well-characterised and widely used for the study of the microvasculature. This will greatly aid the comparison and integration of our results with existing literature.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We do not expect our procedures to cause harm that is more than transient and mild.

We minimise the harms by using genetically altered animals that do not display a phenotype until we induce the genetic modification.

However, should we see any adverse effects from the gene altering substances or the effects of genetic modifications we will increase the frequency of the monitoring, and assess any appropriate measures that can be taken to alleviate any potential unexpected effects.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow LASA, ARRIVE and the NC3Rs resource for Breeding and Colony Management guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will periodically access the NC3Rs website (Including the section entitled "information portal" and "resource library") to learn about the new developments in 3Rs. The FRAME and ECVAM sites can also offer information on potential alternative methods that may become available.

We will periodically perform searches in available repositories (e.g. PubMed) using keywords such as "reduction", "replacement" and "refinement" as well as "mice" or "mus musculus", to identify possible new developments in the 3Rs that could be implemented in our project.

Additional databases (ALTWEB and AWIC) will be periodically consulted to explore alternatives to animal use there could be implemented in the project.

There are also relevant mailing lists and newsgroups (e.g. CompMed and TransgenicList) that can be joined to remain updated on aspects of use of laboratory animals.

We will engage with information provided by the Named Information Officer and a NC3R's regional manager.

60. Therapeutic implications for inflammation- driven 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, therapy, liver inflammation, liver injury, metabolism

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To outline the impact of anti-apoptotic genes in cancer development using models of inflammation- driven cancers (e.g. liver inflammation and cancer).

To determine cellular and molecular mechanisms which regulate inflammation, tissue regeneration in response to injury and cancer development

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Around half of cancer patients in the UK now survive their cancer for more than ten years. But whilst outcomes for some types of cancer have improved enormously, patients with other tumour types continue to do very poorly. And once cancer has spread around the

body, it is still often incurable. An example is given by hepatocellular carcinoma (HCC), a complex tumour of the liver that is most commonly associated with underlying chronic liver inflammation (risk factor).

Genomic analyses have provided a clear picture of the main molecules driving liver tumour initiation and progression. However, only a handful of these drivers are being used as pharmacological targets, which limit their applicability in the clinic. Therefore, there is an urgent need to find additional targets in the pathways that regulate the development of this deadly disease.

Hence, the new insights into how liver cancer develops are expected to have a significant impact on either the prevention (chronic inflammation) or targeted inhibition of the advanced disease. Our goal is to exploit the findings made in animal models of inflammation-driven HCC for rapid translation into the clinic.

What outputs do you think you will see at the end of this project?

Our proposed studies are aimed at a better understanding of the causes and biology of the development of HCC, an incurable tumour of the liver classified as a cancer of unmet need.

Cancer and chronic inflammation represent a considerable health burden in society. A major goal of the proposed program of work is to test the role of key signalling pathways that are implicated in the multistep process of carcinogenesis induced by chronic inflammation.

For example, using GA mice harbouring deletion of anti-apoptotic genes, we can test whether modification of specific signalling proteins alters the ability of generating chemically-induced or spontaneous tumours, in vivo. By studying the impact of perturbing cell signalling pathways regulating apoptosis and cell survival, we can gain insights into the molecular and cellular mechanisms underlying the development of chronic inflammation and cancer. This knowledge might help inform our design of drugs to improve immunity to pathogens (resolution of inflammation) or lead to new insights for potential therapeutic approaches for the treatment of cancer. Given the complexity of the interactions of the inflammatory cells with the tumour microenvironment, in vivo models to study to the impact of signalling pathways are required. However, these experiments will be complimented by detailed analysis of cancer cells responses in in- vitro cultures, as well as biochemical, molecular biology and proteomic approaches.

Important and immediate outputs for the work will include advancement of scientific knowledge in inflammatory disorders and associated development of cancer. The project will help identify novel approaches to manipulate the inflammatory responses. Thus, it is expected that the knowledge gained from our animal experiments will be translated to pre-clinical human studies. A specific benefit would be to understand how inflammation drives the development of tumours with the aim to inform drug companies of the physiological importance of certain anti-apoptotic genes of interest to generate selective drugs to stop tumour development.
In addition, the transgenic mice development carried out in this project licence will be valuable to other scientists interested in developing anti-inflammatory or anti-cancer therapies.

Who or what will benefit from these outputs, and how?

In the short term, the results from our proposed project are directly relevant for academic researchers working in the fields of cancer signalling and drug discovery. The results originating from our research may spur collaborations with pharmaceutical companies which have efficient pipelines to screen for and test drugs or biologicals against putative targets, or available drugs could be repurposed for liver chronic disease and/or HCC therapy.

Our ultimate goal is to improve the health and wellbeing of patients by preventing the formation of liver cancer (via modulating liver injury and inflammation, two major risk factors of HCC development) or by suppressing advanced stages of HCC.

In the long run, it is anticipated that patients would benefit from the insights resulting from our proposed research, which will have sparked translational work by us and other national and international academics through the publication of the results.

How will you look to maximise the outputs of this work?

External dissemination of our results to academics and clinical scientists will be achieved via peer- reviewed publications and presentations at national and international scientific meetings, including hepatology and cancer meetings.

There is a real prospect of translating our findings on novel potential targets for pharmacological inhibition into clinical trials, since several investigators and liver surgeons (collaborating with our team and involved in running national clinical trials for HCC) are interested in the development of improved treatments.

For non-academic users, we propose to communicate our research through a series of Health New outlets as previously realised by the lead investigator and co-investigators. On this regard, the applicant is an active science-communicator at "The Conversation UK", a global media resource providing informed commentaries and debates on the issues affecting our world. He has published several articles in the “Health” section that have been read and shared on social media.

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.

In the proposed work we will use transgenic mouse models to investigate how different protective genes direct the development and progression of chronic inflammation to cancer. Some of these models are already available in our laboratory, while others are becoming available elsewhere, involve the interplay of multiple tissues and factors that cannot be modelled using currently available tissue culture systems.

The availability of resources for genetic manipulation and phenotyping underlies the choice of the laboratory mouse for this work. Over decades of research, this approach has

generated a wealth of knowledge that has formed the basis for a great number of clinical applications in humans. No other species of lesser sentience would fulfil the criteria for this programme of work to the same extent.

Of exceptional importance for the planned studies is the fact that the immune system of mice is well characterized and closely resembles that of the human, which has the important implication that insights from mouse models can be directly translated to humans. Indeed, the mouse and the human genome are the most highly homologous genomes of the large vertebrates.

Specifically, the transgenic mouse models described in this project are required to test whether specific signalling pathways and molecules play a biological and functional role in the in-vivo response to liver injury, regeneration and development of cancer.

In our models we will employ both early young mice (14-days post-birth) and adult (8 weeks-old to 10 months-old) mice given that a precondition for the planned studies is to understand the stages of cancer development from the start of the life throughout the entire of adult life, which mimics the actual timeline of humans being exposed to different lifestyle choice.

Typically, what will be done to an animal used in your project?

The proposed study will employ genetically modified animals (GMAs) and therefore tissue biopsies will be taken by ear punch and subjected to molecular genotypic analysis to determine the genetic status.

Some mice will be administered substances, such as hepatotoxin (i.e., CCl4 or TAA) by intraperitoneal (most cases) or intravenous (rare cases) injection, in drinking water or via diet supplementation. Some mice will receive a single (acute, high) dose of hepatotoxin to test the acute response to liver injury within 48-72 hours post-administration. Some other mice will receive a repetitive (usually once a week for up to 6 weeks) (chronic, low) dose of hepatotoxin to test the regenerative capacity of the liver.

A typical experiment consists of injecting carcinogens to 14-days old mice and when adult they will be freely put on either standard diet or a modified diet with high-content of fat, to check how the combination of carcinogens (first hit) and the different lifestyle choice (second hit) would impact the development of cancer in certain GM animals. Following this regimen for up to 10 months of age, animals will gradually develop tumours (tumours at this stage are clinically silent and therefore only a low percentage of animals are expected to show clinical signs of disease)

Small amounts of blood may be taken from a superficial vessel (e.g. tail vein) throughout the experiment. The purpose of the blood withdrawal is to determine the blood sugar content in some experimental mice.

Administration of adeno-associated virus-mediated expression of cDNA may be also used to deliver ectopic cDNA directly in certain type of cells (i.e. liver cells) without apparent damage to the target tissue

In certain experiments we may also employ the administration of substances such agents that label proliferating cells (e.g. BrdU, EdU) or drugs (chemical compounds) that rescue (alleviate) the symptoms of liver injury induced by liver toxins and/or diet.

What are the expected impacts and/or adverse effects for the animals during your project?

Breeding and generation of genetically modified animals (GMAs) that will be used are generally vital and present no spontaneous development of disease. Therefore, GM animals bred and produced are not expected to exhibit any harmful phenotype.

Animals with altered immune status will be maintained in a barrier environment thereby minimising the likelihood of compromising health.

After injection of carcinogens some animals have the potential to develop a harmful phenotype, eg tumours, neurological signs, after a certain age (over 12 months). However most of the experiments will be terminated up to 10 months of age and therefore administration of these substances will have minimal impact on their wellbeing.

Administration of hepatotoxin (i.e., CCl4 or TAA) causes hepatic necrosis. From previous experience we know that the vast majority of animals tolerate single acute dose for up to 72 hours or multiple (chronic) intraperitoneal injections for up to 6 weeks of CCl4 or TAA. There is the possible development of pyrexia and weakness without local pain (<1%). During chronic studies, the animal may develop jaundice (more obvious in albino animals) and ascites. However, the liver should not fail at the doses used and at no time will signs of hepatic encephalopathy occur (such as incoordination, circling movements and being comatose).

The adverse effects of dietary interventions could be the development of diabetes, in which liver may be involved. Longitudinal measurement of body weight and blood glucose test can pick up changes at an early stage thus implementation of humane endpoints can be done accurately.

Administration of tissue labelling substances such as BrdU, do not lead to any unfavourable adverse effects.

Administration of adeno-associated virus-mediated expression of cDNA is used to deliver ectopic cDNA directly in certain type of cells (i.e. liver cells) without apparent damage to the target tissue. We have extensive experience in this technology, and have not observed significant adverse effects of adenoviruses in livers.

All intra-peritoneal injections will be carried out by experienced personal licence holders and animal will be monitored for any signs of pain post-injection.

In all cases general clinical signs (such as Inactivity, Isolation from cage mates, Pinched face, narrowing of the eyelids, Discharge eyes/nose, reduced grooming, Scratching, Abnormal breathing, reduced food or water intake, Hunched posture, boarding of abdomen; aggressiveness, abnormal Body weight changes) will be used to indicate the wellbeing of the animals. Any animal that displays one or more of the above clinical signs will be immediately killed by a Schedule 1 method if its condition threatens to exceed the moderate severity limits in place in the experimental protocols of this licence.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

For mice breeding and maintenance it is expected to have a mild severity (20% of cases) and sub- threshold (80% of cases).

All the experimental protocols fall under the category moderate severity (30% of cases) since a fraction of mice may develop inflammation followed by induction of liver fibrosis and generation of tumours (either induced by carcinogens or following an injury). The remaining 70% would experience a mild severity.

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?

Our aim is to understand complex biological processes in primary cells, as this information will be most relevant to the design of future therapeutic approaches. Whilst some basic questions can be addressed using immortalized cell lines, such approaches have numerous limitations, most importantly that these cells are by definition quite distinct from primary cells. Immortalized cell lines frequently have oncogenic mutations in key signalling proteins, such as tyrosine kinases or small GTPases, which enable their survival and growth in vitro. As such, these alterations in key cell signalling proteins render the analysis of signalling transduction in cell lines as quite distinct to primary cells.

Wherever possible, we employ ex vivo techniques to study immune cell function; e.g. co-culture of lymphocyte populations with tumour cells in vitro. However, to understand the complex interplay between immune cells, cancer or toxic agents requires the use of in vivo models. Indeed, the complex cell-cell interactions that govern the initiation, amplification and resolution of an inflammatory response cannot be studied outside of an animal and only jawed vertebrates have B and T lymphocytes, which play a central role in regulating this response.

Therefore, for much of the proposed work we will use mouse models to investigate how different protective genes direct the development and progression of chronic inflammation, immunity and cancer. These diseases involve the interplay of multiple tissues and factors that cannot be modelled using currently available tissue culture systems.
With this in mind and to best of our knowledge, there are currently no non-sentient alternatives to the use of animals for our work.

Which non-animal alternatives did you consider for use in this project?

For some aspects of our study regarding the gene/pathway validation studies as well as e.g. the investigation of the deregulated function of proto-oncogenes, whenever possible

we plan to investigate those at the molecular level using appropriate in vitro cultures of human cell lines.

In complementary studies we are also employing the use of hepatic organoids, functional three- dimensional (3D) in vitro models of the liver that may serve as a novel platform to address diverse research questions pertinent to hepatic development and regeneration, detoxification and metabolism studies, liver disease modelling, and adult stem cell biology.

Why were they not suitable?

Until now, no functional in vitro cell culture system has been developed that faithfully mimics all aspects of the in vivo liver reaction. A major caveat is that the in vitro cultured cells do not mimic the microenvironment of the liver cells within its tissue. Thus, in the absence of an in vitro system for the highly complex process of liver cancer development, the use of animal models remains the only rational approach to study the distinct stages of chronic inflammation and cancer development in the context of the complex living organism.

Liver organoids have major limitations that include: a lack of organotypic features, as compared to in vivo (i.e., the lack of inflammatory cells, the lack of the perisinusoidal space (or space of Disse) where hepatic stem cells would reside, along with a non-physiological ratio of hepatic stem cells to hepatocytes (HSC making up 50% of the spheroids, compared to 5–8% observed in the liver)

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Our previous experience allows us to ensure that animal usage is kept to a minimum and experiments designed in such a way that we expect the outputs to be measurable and reproducible. Established breeding colonies are, where required, maintained as homozygous lines such that all animals produced are of the desired genotypes.

The proposed experiments are based on the decade-long experience in the administration of hepatoxins to induce these models. We therefore do not anticipate carrying out pilot studies. However, should less invasive substances for the induction of fibrosis or its modulation become available during the project, we will determine the suitability of those substances by conducting small scale pilot studies and comparing results with our established protocol.

In order to achieve statistical significance and experimental reproducibility we typically perform an establishment (pilot) study by using small number of mice (typically 5-10 mice per group). This will help us to keep the animal numbers at minimum while testing the value of our experimental protocol.

Subsequently, we perform an efficacy study and the total number of animals is determined by G-power calculation, based on similar studies performed in other GA models. The numbers of mice to be maintained and used in experiments will be kept at an optimal minimum to ensure that reliable experimental data is obtained.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

For all experiments, a randomized block design approach will be used and scoring will be “blinded” to the user. We will choose animals of similar weight and age. All the experiments will be performed in a ‘clean’ and a non-stressful environment reducing the risks of clinical or sub-clinical infection. All our animals are of the same genetic background, therefore, we will control the genetic variation using inbred strains.

In all experiments we will refer to the Experimental Design Assistant (EDA) available on the NC3Rs website.

Moreover, we follow the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines to ascertain that our research design is compatible with the ARRIVE checklist of recommendations.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Our project includes efficient breeding to reduce the number of GMAs that are not of the desired genotype, which is a general caveat of the interbreeding of multiple alleles. For example, in the experiments with the experimental cohort of geneXfl/flgeneY-Cre (tissue specific knockout), geneXfl/+geneY-Cre and geneX+/+geneY-Cre, we follow a breeding strategy in which geneXfl/fl mice are intercrossed with geneXfl/+geneY-Cre mice to obtain Cre-expressing homozygous and heterozygous mice for the floxed allele, and geneX+/+geneY-Cre with geneX+/+ mice to obtain the Cre- expressing control mice. This strategy, which is possible to follow since all mouse lines are on the C57BL/6 genetic background, considerably reduces the generation of ‘unwanted’ genotypes that would have to be humanely culled.

Use of non- invasive in vivo imaging can also pick up progression of pathological changes in longitudinal study in one animal. This allows consistency of humane endpoints, thus increasing experimental reliability and reduction of animal use.

Although the majority of our experiments are focusing on the liver response to injury, we will systematically collect most of the tissues in a single animal. This will allow us to have a tissue bank collection of animals following these protocols that can be shared with other groups and investigators.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

During the past 5 years we have generated the above GAA animal models and refined the experimental methodologies and found that 70% of all mice used on this project will fall under mild severity. The majority of animals produced under the breeding program will be used to supply tissues for in vitro analyses. The majority of mouse lines have no defects beyond alterations in or loss of immune cell populations and suffer no ill effects in an SPF animal facility.

New immune/inflammatory challenges might provoke unpredicted responses; pilot studies to assess possible adverse consequences are undertaken in these circumstances. For each protocol, specific details for minimizing animal suffering are given. In all cases, we will refer to the published literature for each model to minimize suffering to mice. For all procedures, we will apply the least invasive methods of dosing and sampling appropriate to the objectives of the experiment, including the use of anaesthesia for humane restraint where necessary. For example, in animals with tumours induced by chemicals, tumour growth will be assessed by IVIS imaging of tumour cell bioluminescence.
Sequential imaging of animals in longitudinal studies can pick up pathological changes at an early stage thus implementation of humane end-points can be implemented more accurately, and consistently resulting in refinement of experiments.

Why can’t you use animals that are less sentient?

The availability of resources for genetic manipulation and phenotyping underlies the choice of the laboratory mouse for this work. Over decades of research, this approach has generated a wealth of knowledge that has formed the basis for a great number of clinical applications in humans. No other species of lesser sentient would fulfil the criteria for this programme of work to the same extent.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

After administration of substances, animals are closely observed for adverse effects, and monitored daily for signs of ill health such as piloerection / hunched posture / lack of appetite / reduced activity. Animals that are subjected to procedures with potential adverse effects will be monitored more frequently as described within the protocol steps. This will allow prompt application of humane endpoints if required.

Animals on longer term studies will undergo an acclimation period of at least a week during which time they will be handled regularly.

Use of non- invasive in vivo imaging can also pick up progression of pathological changes in longitudinal study in one animal. This allows consistency of humane endpoints, thus increasing experimental reliability and reduction of animal use.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow the most recent Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines, which have been published:

The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research, PLoS Biol 18:e3000410, 2020; PMID: 32663219; PMCID: PMC7360023.

We also make use of the most recent updated guidelines on the welfare and use of animals in cancer research:

Workman P, et al., Guidelines for the welfare and use of animals in cancer research. Br J Cancer. 2010 May 25;102(11):1555-77.

For guidance on administration of substances we follow the LASA GOOD PRACTICE GUIDELINES Administration of Substances (Rat, Mouse, Guinea Pig, Rabbit): https://researchanimaltraining.com/wp-content/uploads/2021/05/lasa_administration.pdf

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will make use of the NC3R website and available 3R online resources. Through our connection with other local and national research groups, we have the opportunity to discuss protocol-relevant issues that may benefit our protocols.

61. Nutrient metabolism in ruminants

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Nutrient, Metabolism, Ruminants

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?

The purpose of this programme of work is to advance scientific understanding and knowledge of the digestive function and nutrient metabolism of ruminants.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The extent to which nutrients in feed are converted into milk and meat by ruminants directly impacts the environmental impact of animal agriculture, animal health and welfare, and the nutritional quality of milk and meat from ruminants. This project will conduct studies that will form the basis of improved feeding strategies that optimise nutrient supply and utilisation for milk and meat production with minimal environmental impact and optimal composition for consumers.

What outputs do you think you will see at the end of this project?

The main outputs of this project will be the generation of new knowledge on understanding the digestion of ruminant animals and how nutrients are used efficiently for growth and lactation.

Who or what will benefit from these outputs, and how?

Short-term benefits

New knowledge and data will be made available to other scientists through publication in open-access peer-reviewed scientific journals and presentations at national and international scientific conferences and meetings. Publications will be publicly available through a central archive. Under the previous project licence, we published 35 papers (6 more in preparation) and a similar number of short communications and abstracts presented at multiple international and national scientific meetings.
Dissemination of the new data will contribute significantly to the database on ruminant digestive physiology and contribute to an integration of knowledge between several related fields of study.

Medium-term benefits

Data generated from studies of energy and nitrogen balance will be used to develop and revise models for predicting greenhouse gas emissions from animal agriculture. Improved emission factors for methane and nitrous oxide in national and international greenhouse gas inventories have the potential benefit of monitoring changes in emissions in response to mitigation strategies.

Long-term benefits

Research findings have the potential to contribute to the sustainability of the UK livestock sector and the well-being of the UK population. Identifying improvements in digestive and metabolic efficiency could have benefits to livestock producers through lower production costs and reduced environmental

pollution from greenhouse gases such as methane and nitrous oxide, thereby reducing the carbon footprint of milk and meat products. Improvements in nitrogen use efficiency have the potential to protect the natural environment by reducing slurry and fertiliser nitrogen application to farmland, thus decreasing the likelihood of a major problem from run-off and subsequent eutrophication of ground and surface water. Understanding how the composition of milk is controlled and changed by manipulation of the animal’s diet could provide the consumer with animal products of improved nutritional specification. Milk and milk products with reduced fat and saturated fatty acid content could substantially reduce the risk of cardiovascular disease, the metabolic syndrome and other chronic diseases associated with the consumption of animal-derived foods, particularly dairy products. Work on diverse forages has the long-term potential to harness the benefits of mixed species swards to be more productive under variable climates (drought and flood), less reliance on nitrogen fertiliser due to nitrogen fixation capabilities of legumes and improve biodiversity and soil structure compared to conventional ryegrass.

How will you look to maximise the outputs of this work?

The ARRIVE guidelines 2.0 will be followed in all publications.

We collaborate with research groups in Europe and North America, exchanging data, results and best practices. We have strong and effective existing links engaging with policymakers in government, supermarkets and the UK livestock industry through established pathways to impact including:

- Hosting approximately events for visiting groups where attendees learn about the research being conducted. These include academics, farmers, policymakers, industry representatives, consultants and students, as well as the general public
- Featuring in national and local news providers and trade journals
- Developing best-practice guidelines
- Maintenance of informative websites
- Working with industry to promote the adoption of research findings
- Embedding knowledge within teaching
- Working with charitable organisations and academic societies

We have firm commitments to the aims and principles of Open Research and promote the publication of unsuccessful research in appropriate, publicly available databases.

Species and numbers of animals expected to be used

- Cattle: 1000
- Sheep: 100

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Digestive physiology and metabolism in ruminants are unique and therefore models based on other species are not applicable. The animal models chosen are the commercially important species for the UK livestock and food supply industries and they are the major sources of environmental pollution from livestock farming. Data for the proposed species has been widely reported in the scientific literature and the use of similar species will allow direct comparison of our results and reduce the need to replicate some investigations for which data already exist.

Typically, what will be done to an animal used in your project?

Typically, on protocol 1, animals will be subjected to an altered diet. During this time, animals may be blood sampled by venepuncture or temporary indwelling cannula and/or be stomach tubed to obtain samples of rumen contents. Both of these procedures will cause only mild and transient pain.

Where appropriate cattle may also be restrained in open-circuit respiration chambers for periods of up to eight days, with rest intervals of at least two weeks between periods in the chambers. During periods in the chambers, animals will be held in individual stalls (large cattle), pens (calves) or crates (sheep) and may include the total collection of urine and faeces.

Typical within-animal design studies (e.g., Latin square) will last 12-16 weeks with respiration measurements and/or blood/rumen samples taken during every third or fourth week, before a four- week rest period, with no more than three experiments per 12-month period.

At the end of the study, these animals will be kept alive and rehomed to the University of Reading commercial farm for subsequent re-use, milk/meat production, sale to another farm or sent for slaughter subject to veterinary examination.

A small number (4-6 animals per year) will be used on protocol 2 and have surgery to create a permanent fistula in the left flank, which via a permanent cannula will allow access to the rumen. The fistula will be used for collecting samples of rumen contents (solid, liquid and gas), the introduction of inert markers, incubation of test material in situ, mounting test monitoring equipment on the cannula bung and for administration/withdrawal of rumen boluses. Cattle that have rumen fistulae created will suffer moderate pain during the surgical procedure and for a period of up to two weeks afterwards. To mitigate this, analgesia will be used and the surgery will be carried out under local or general anaesthesia by experienced veterinary surgeons or personal licensees using aseptic techniques.

Appropriate post-operative analgesia will be given. Following recovery, these cattle suffer no further adverse effects and will live a typical commercial farm life. Animals remain in very good bodily condition and may become pregnant, give birth and produce milk as in typical commercial dairy production. Because of their surgical preparation, animals will be kept alive and re-used in different studies, up to a maximum of six studies per 12-month period. At the end of their working life, they will be euthanised via a Schedule 1 method.

At the end of procedures, animals may be:

1. Re-used subject to veterinary examination certifying that previous use has not compromised the biological integrity of the animal; previous use has not rendered the animal unsuitable for subsequent use and the cumulative suffering experienced by the animal during its lifetime has not exceeded the moderate level of severity.

2. Rehomed to the University of Reading commercial farm subject to veterinary examination as above and that the animals have been suitably socialised.

3. Killed by a Schedule 1 method.

4. Killed by a non-schedule 1 method - Penetrative captive bolt device. Severe and irreversible damage of the brain provoked by the shock and the penetration of a captive bolt followed by the destruction of the brain or exsanguination. The loss of consciousness and sensibility shall be maintained until the death of the animal.

What are the expected impacts and/or adverse effects for the animals during your project?

Cattle will have surgery to create rumen fistulae, which are similar to a colostomy in people but in the forestomach. They are expected to recover quickly and will be given painkillers and post-operative care the same as people recovering in a hospital.

Animals are provided with all needs to achieve their full biological potential and housed in appropriate facilities with environments to promote natural behaviours and animal interactions. Animal health and welfare are monitored daily and any animal exhibiting any adverse effects will be removed from the study and veterinary advice sought. The longevity of animals at our facility can be more than double the average longevity of similar animals on a farm.

Expected severity categories and the 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: 97% mild; 3% moderate
Sheep: 100% mild

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?

The work under this project is principally concerned with the whole animal system from dietary input to proteinaceous products (milk/meat) and as such there are only limited opportunities to exploit alternatives to the use of live animals.

Which non-animal alternatives did you consider for use in this project?

The processes of nutrient digestion and utilisation within ruminants have been simulated using both in vitro and modelling techniques. We have established an in vitro laboratory and a biomathematics group to provide these techniques and expertise, so permitting the replacement of experimental animals. As appropriate, in vitro techniques will be used to evaluate the nutritional quality of feedstuffs for digestibility, and their ability to supply nutrients. In vitro, techniques attempt to mimic the anaerobic, hydrolytic and reductive environment of the rumen and will be used to provide kinetic data describing the extent and rate of degradation of a complete diet or dietary component by the rumen microorganisms over time. In vitro, techniques will also be used to study the rate of volatile fatty acid production during fermentation and the rate of biohydrogenation of unsaturated fatty acids. Where applicable, in silico biomathematical models of rumen fermentation will be used to predict changes in the pattern and quantity of fermentation products in response to specific perturbations in dietary input, for example, observing the changes in volatile fatty acid and methane production in response to changes in the supply of cellulose/hemicellulose and starch to the rumen. Other models are used to predict diurnal patterns of nutrient metabolism in the rumen, for example, volatile fatty acids, rumen pH, hydrogen and methane production. Kinetic models of amino acid metabolism across

tissues are used to predict amino acid requirement relative to the synthesis of export proteins, constitutive protein turnover and catabolism. For example, predicting changes in milk protein output in response to changes in the supply of amino acids to the mammary gland and the differential response in milk protein synthesis versus that required to maintain constitutive protein turnover in the mammary gland.

Why were they not suitable?

While every effort will be made to develop and utilise in vitro and in silico modelling techniques, they cannot adequately represent the complex interactions between rumen fermentation, digestion, metabolism and the endocrine regulatory systems; therefore, many of the procedures detailed still require the use of animals, for example, lactation responses.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimated numbers of animals to be used are based on previous licence usage, undertaking similar research programmes.

Studies of the type envisaged for large animals (cattle and sheep) are typically “within animal” experimental designs (e.g., Latin square, crossover or switchback) with four to six-week periods. Such designs allow animal variation to be accounted for in the statistical analysis, thereby minimising the number of animals required. “Between animal experiments” may also be used, e.g., randomised-block designs and these require larger numbers of animals than within animal experiments.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will use available resources to assist with experimental design including the NC3Rs Experimental Design Assistant, the ARRIVE guidelines 2.0 and the SmartCow book of techniques (Methods in cattle physiology and behaviour research – Recommendations from the SmartCow consortium, available online at https://books.publisso.de/en/publisso_gold/publishing/books/overview/53/186/about). Experimental designs are discussed by the PPL holder, NACWO, NVS/VS and other users prior to commencement to enable the most refined trial design.

To successfully undertake such a research portfolio, SMART objectives will be identified and applied to all experimental work. Although several designated procedures under the Act will be employed, opportunities to reduce animal numbers without compromising accuracy will be explored on a study- by-study basis. For each programme of work, the statistical issues concerning (i) sample size, (ii) study design and (iii) data analysis will need to be addressed. This will often be done in consultation with a consultant statistician. Where there are several different component experiments, then statistical advice may also

be sought to identify how the studies could be designed to get as much information as possible from across the studies. Examples of the above statistical issues and how they might be addressed are as follows:

When planning an individual study, coefficients of variation (or estimates of random variation) from previous studies for the key response variables will be used to conduct sample size calculations that ensure optimum numbers of animals are used in the study, so that a response can be identified as being statistically significant with a high level of statistical power.

Studies of the type envisaged for large animals (cattle and sheep) are typically “within animal” experimental designs (e.g., Latin square, crossover or switchback) with four to six-week periods. Such designs allow animal variation to be accounted for in the statistical analysis, thereby minimising the number of animals required. Often these studies have about four to eight animals in them. “Between animal experiments” may also sometimes be used, e.g., randomised-block designs and these require larger numbers of animals than within animal experiments.

Depending on the type of study, the data collected will be analysed using statistical methods relevant to the study design. For example, general linear models with effects for animal, period and treatment will be used for within animal investigations. For between animal studies where data are collected repeatedly over time, mixed model analysis may be used to test the effects of treatments over time.

The re-use of animals, subject to the criteria detailed in the project plan, will reduce the number of naïve animals required to complete the programme of work.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

To optimise the number of animals used, in vitro techniques will be used to evaluate the nutritional quality of feedstuffs for digestibility, and their ability to supply nutrients. In silico biomathematical models will be used to predict fermentation products and protein and amino acid requirements relative to predicted production responses.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Digestive physiology in ruminants is unique and therefore models based on other species are not applicable. The animal models chosen are the commercially important species for the UK livestock and food supply industries and they are the major sources of environmental pollution from livestock farming. Data for the proposed animals have been

widely reported in the scientific literature and the use of similar animals will allow direct comparison and augment the existing data set.

This programme of work will involve nutritional trials. For example, the level of dietary protein, and protein from different sources will be used as part of the typical diet fed to ruminants. In addition, ruminants will be given feed supplements as part of their diet. These interventions are likely to change metabolism in the ruminal microbiome and/or post-absorptive nutrient/mineral metabolism in the animal which could modify production responses and excretion of environmental pollutants. Nutritional intervention studies are highly unlikely to cause lasting harm to ruminants. There will be moderate discomfort post-surgery that will be mitigated via pre and post-surgical analgesia.

A re-design of the main area of cattle accommodation in the metabolism unit allows rumen fistulated animals once permanently housed in tie stalls to be housed, where appropriate, in a commercial free- stall yard allowing freedom of movement and social interaction. Animals off trial are allowed access to pasture loafing areas when weather permits. New loose yards also allow animals to be housed in groups bedded on straw where appropriate. The restrictiveness of long-term housing for cattle can now be tailored to the requirements of individual studies reducing periods of individual restraint to periods of specific experimental protocol requirements.

We have redesigned the tie stalls for cattle restraint in the metabolism unit, providing animals with a larger individual area and allowing lateral restriction to be applied and released as necessary rather than being constantly in place in the previous design. A mobile feed trough allows for simple bed length adjustments enabling tie stalls to be tailored to individual animal sizes and a larger capacity drinking trough rather than the previous press-fill drinking bowls allow for more natural drinking behaviour.

We were partners in a key European cattle research infrastructure project (SmartCow) that evaluated and refined state-of-the-art in vivo methods in the field of nutrient use efficiency of cattle to improve their accuracy and reduce the number of experimental animals used and develop possible proxies (biomarkers) and less-invasive methods to reduce constraints on experimental animals and increase phenotyping capabilities for feed efficiency and its components, including methane emissions, in cattle. As part of the project, collection techniques for faeces and urine were refined as described in the open-access SmartCow book of methods. Methods in cattle physiology and behaviour research – Recommendations from the SmartCow consortium available online at https://books.publisso.de/en/publisso_gold/publishing/books/overview/53/186/about

Other recent refinements include the use of water beds in the respiration chambers to increase animal lying time and the adoption of the GreenFeed system for the measurement of methane production as an alternative to respiration chambers when only methane measurement is required.

Why can’t you use animals that are less sentient?

Using animals of lower sentience would not help achieve the programme’s aims due to the unique physiology of ruminant digestion and metabolism. For example, the rate of gluconeogenesis in non- ruminant animals is lowest after feeding and highest during an energy de□cit; in ruminant animals, the rate of gluconeogenesis is highest after feeding. Because of rumen fermentation, ruminant animals derive far more energy from □brous plant-based material and volatile fatty acids than non-ruminant animals and likewise, they

obtain far more of their amino acid requirements from non-protein nitrogen and ammonia through microbial protein synthesis than non-ruminant animals. Lipid digestion in ruminants is distinctive and the rumen environment results in glycerol from triacylglycerols and phospholipids being fermented to volatile fatty acids and unsaturated fatty acids are hydrogenated to mostly saturated fatty acids before absorption. In ruminants, fatty acids are primarily synthesised in adipose tissue using rumen-derived acetate as the main carbon source due to low dietary glucose availability, whereas in rodents, fatty acids are mainly synthesised in the liver, relying on glucose carbon for lipogenesis.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

As in commercial best practice, animals are managed to increase lying time. Water beds achieved this for the periods spent in the respiration chambers. During urine collection traditionally, a bladder catheter was used to divert urine into a container, on occasions this caused discomfort and infection.

To perform the same task a separator was developed which attached round the vulva with glue, this system worked well with cows in average body condition but caused hair loss around the glued area. Animals were restrained while the separator was being attached. We now collect both faeces and urine using a simple chute attached to the cow with a light equine harness, and separate faeces and urine mechanically post-collection. This system causes less discomfort to the animal especially when collections are repeated relatively frequently.

Animals are pre-trained to the respiration chamber and enter in pairs and are visible to each other. Training for the respiration chambers and chute collection system takes place over several days using feed enticement and food rewards. The length of time the animal spends in the facility is gradually increased, initially with doors open and finally with doors shut once they are comfortable with the surroundings. During training for the respiration chambers, animals are also pre-trained for the chute system to enable the total collection of urine and faeces. The equipment is introduced gradually over several days using food rewards. Initially, the girth strap is fitted and animals are allowed time to adapt, followed by the girth strap with associated supporting straps and finally the complete chute system. Animals are only allocated to study when they are observed to be comfortable with the equipment and surroundings, are seen standing and lying down at will and intake and production are at pre-restraint levels.

Further refinements to procedures as they occur will be incorporated into experimental protocols that describe the care and use of animals under this project.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use the NC3Rs website including the Experimental Design Assistant, the ARRIVE guidelines 2.0 and the SmartCow book of techniques; Methods in cattle physiology and behaviour research – Recommendations from the SmartCow consortium, available online at https://books.publisso.de/en/publisso_gold/publishing/books/overview/53/186/about

Best practices for large and small ruminants are shared with, and between, our national/international collaborators, and procedures are adapted and implemented where appropriate to improve and refine the animal models used.

Technical staff have an established track record of working with ruminants and have completed successful trials on large and small ruminants under the applicant's previous project licences. Experiments are discussed by the project holder, NACWO and NVS/VS before commencement to enable the most refined trial design.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The applicant consults the monthly NC3Rs newsletter and notices from LASA and LARN distributed by the establishment's Named Information Officer. The applicant maintains awareness of advances in the 3Rs from discussions with collaborators and during the previous 12 months, has attended project licence refresher training, LARN meeting and a statistics workshop. Amendments will be made to the project's animal trials where relevant and appropriate. The Animal Science research group has collaborative contact with research groups in the UK, North America and Europe, working with ruminants, and advances in techniques and practices are shared between institutions. We have advised researchers who have since adopted and modified our practices to suit their specific purposes.

62. Cerebrovascular and inflamm-ageing link to neurodegeneration and dementia

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

vascular biology, blood-brain barrier, pericyte, brain imaging, dementia

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this project is to understand how cerebrovascular dysfunctions contribute to dementia. We propose to investigate the mechanisms triggering cerebrovascular failure with a particular focus on the two major brain vascular cell types, endothelial cells and pericytes. Importantly, greater knowledge in these areas may lead to novel therapeutic targets for cerebrovascular protection in chronic neurodegenerative diseases, particularly 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?

Dementia is becoming an increasingly important disease due to an ageing population and limited treatment options. Cerebral small vessel disease (SVD) and Alzheimer's disease (AD) are the two most common causes of dementia with vascular dysfunction being a large component of both their pathophysiologies. The neurogliovascular unit (NGVU), and in particular the blood-brain barrier (BBB), is required for maintaining brain homeostasis. A complex interaction exists between the endothelial cells, which line the blood vessels and pericytes, which surround them in the NGVU. Disruption of the BBB occurs in dementia precipitating cognitive decline. The mechanisms which drive endothelial cell and pericyte dysfunctions are largely unknown, with no established therapies.

What outputs do you think you will see at the end of this project?

This project has broader relevance to dementia, in particular cerebral small vessel disease (SVD) and Alzheimer's disease (AD), where pericytes were shown to die early. The identification of modifiable pathways which contribute to vascular dysfunctions is a major focus of global efforts to tackle the vascular contribution to dementia. The outcome of this project will shine light on the cellular and molecular underpinnings of vascular dysfunctions leading to pericyte death and cerebrovascular breakdown. This will not only help to better understand the early pathophysiological processes, ultimately triggering neurodegeneration and dementia, but also help designing vascular-targeted treatment strategies to protect the brain in SVD and AD, and possibly other neurological disorders.

Who or what will benefit from these outputs, and how?

Our project has the potential to influence clinicians and scientists in the fields of vascular biology, pericyte biology, ageing, cognition, and neurological/cardiovascular disease. Primary academic outputs from this project will be made immediately available through open access publications and through presentations at conferences. Reagents and tools generated will be made available to research groups. Although pericytes are increasingly investigated in the context of brain disorders, there is a critical knowledge gap that our project will aim to fill out within the next 5 years. Only recently, pericyte and blood-brain barrier (BBB) dysfunctions were found to play an early and important role in the development and progression of several neurological disorders including Alzheimer's disease (AD) and cerebral small vessel disease (SVD). Our project has a strong interdisciplinary focus which may influence researchers in several disciplines, by identification of new cellular and molecular targets for intervention in cognitive decline, and by use of novel approaches as described below.

1. Researchers in the field of neuroimaging

We will apply, develop, and optimize brain imaging methods using cutting-edge magnetic resonance imaging (MRI) and positron emission tomography (PET) in animal models relevant to AD and SVD. We will be focusing on vascular, immune, and neuronal functions and malfunctions that are highly relevant to a large number of brain disorders. Also, this technology has the capability of imaging the whole body which would be of interest for researchers of many field including multiple sclerosis (spinal cord imaging for instance), cancer, cardiovascular disease, inflammatory bowel disease, and so on. Imaging protocols and processing will be made available to academic beneficiaries. Of note, the preclinical imaging tools can be translated into clinical research which will broaden researchers' interest across the UK and worldwide.

2. Researchers in the vascular field

This work can also influence researchers by scientific innovation. This will be the first use of a brain pericyte-specific deficient mouse model to investigate vascular, immune, neuronal, and cognitive functions in a context of dementia. One of our goal is to uncover the molecular mechanisms by which endothelial cells regulate pericyte functions via transcriptomic analyses of different mouse models relevant to AD and SVD. We will provide targets and pathways of interest which can be applied to the investigation of other diseases involving vascular defects.

How will you look to maximise the outputs of this work?

We will maximise the outputs of our project by making all the data available to academic beneficiaries as we have always done in the past, through i) presentations at national and international conferences, ii) participation in local meetings with other groups with overlapping research interests (in brain imaging, vascular biology, and neurodegeneration), iii) making imaging protocols (for MRI and PET) as well as homemade software packages publicly available, and iv) ensuring timely publication in open access journals and in preprint journals when possible.

Species and numbers of animals expected to be used

- Mice: 10000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Animal models are required to understand and dissect the complex mechanisms by which vascular inflammation leads to pericyte and blood-brain barrier (BBB) dysfunctions, and how pericytes in turn destabilizes the endothelial cells lining the BBB. Manipulation of brain vascular cells (i.e., endothelial cells and pericytes) can only be meaningfully studied in the mammalian brain, as they rely on specific cell interactions that are temporally and microenvironmentally restricted. To date, experimental systems that allow this are limited, and must involve vertebrate animals. This is particularly true with respect to the proposed project, which will involve the physiological and genetic assessments of vascular cells and functional measures of BBB in relation to brain/cognitive functions.

The use of genetically altered mice is critical to investigate how endothelial and pericyte dysfunctions contribute to neuron loss and cognitive decline. The mouse strains chosen are partly based on data from human cerebral small vessel disease (SVD) and Alzheimer's disease (AD) brain tissue, ensuring their disease relevance. Our new brain pericyte-specific mouse model will allow both longitudinal tracing and genetic manipulation of brain pericytes. In addition, a chronic pericyte-deficient mutant will be studied in parallel. These important strains will allow us to mimic brain pericyte loss, a key and early feature of both SVD and AD pathobiology. Furthermore, we will move from this reductionist approach to identifying vasoprotective strategies to testing them on transgenic SVD and AD mouse models. All the mice will be studied at the mature adult stage to be one step closer to mimicking the human conditions in elderly.

Typically, what will be done to an animal used in your project?

Animals will undergo magnetic resonance imaging (MRI), positron emission tomography (PET) and/or two-photon laser microscopy procedures under anaesthesia at different time points. They will receive intravenous injections of MR contrast agents, radiotracers and/or fluorescent probes. A subset of mice will have a cranial window on top of their frontal cortex for live two-photon imaging. In parallel, a panel of cognitive tests will be performed longitudinally. These imaging and behavioural procedures will be followed by euthanasia and brain/biofluid tissue collection.

What are the expected impacts and/or adverse effects for the animals during your project?

The adverse effects are based on our experience in the last several years. First, there are minimal adverse effects for the transgenic lines. They all develop chronic vascular dysfunctions which include subtle and local vessel leakages, followed by subtle dementia-like deficits in neuronal and cognitive functions. All the imaging procedures will be performed under anaesthesia with continuous monitoring of anaesthetic gases, breathing rates, cardiac rhythms, and temperatures. Animals will receive intravenous injections of contrast agents/radiotracers that are given every day in clinical practice. We do not expect adverse effects related to the imaging procedures that are mild and transient. Animals with cranial windows are monitored daily after surgery and then weekly to ensure they feel well and receive the appropriate level of care. Although not expected, if an animal exhibits moderate or sustained features of illness (e.g., weight loss > 20%, hunching, altered behaviour, or any signs of discomfort, distress, pain, etc) that persist for 48h, it will be removed from the study and humanely euthanised.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We do not expect adverse effects related to the imaging procedures that are mild and transient. The potential adverse effects are associated with anaesthesia, surgery, and cranial window placement. Deaths resulting from anaesthetic are uncommon (< 1%), and will be minimized by ensuring correct dosing of anaesthetics and by good monitoring of breathing rate, cardiac rhythm, and temperature. We do not expect any kind of infections. Also, it is possible to have window breach in < 10% of the animals. If this is the case, the animal will be removed from the study and humanely euthanised.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animal models are required to understand and dissect the complex mechanisms by which vascular inflammation leads to pericyte and blood-brain barrier (BBB) dysfunctions, and how pericytes in turn destabilizes the endothelial cells lining the BBB. Manipulation of brain vascular cells (i.e., pericytes and endothelial cells) can only be meaningfully studied in the whole animal, as they rely on specific cell interactions that are temporally and microenvironmentally restricted. To date, experimental systems that allow this are limited, and must involve vertebrate animals. This is particularly true with respect to the proposed experiments, which will involve the physiological and genetic assessments of vascular cells and functional measures of BBB in relation to brain/cognitive functions.

Which non-animal alternatives did you consider for use in this project?

For some physiological, genetic, and pharmacological screening related to the core objectives of this project, in vitro preparations, such as cell cultures will be used. For instance, we plan to use blood- brain barrier (BBB) in vitro systems made of endothelial cells, pericytes, and microglia. However, in vitro methods can only provide a limited replacement as the hypotheses generated in vitro need to be verified in real organisms.

Why were they not suitable?

Because the nature of this project is in vivo investigation of vascular dysfunctions underpinning neuronal and cognitive alterations in the context of dementia, in vitro systems are not adequate. Vascular-mediated neuronal and cognitive responses cannot be fully recapitulated using in vitro models without using valid animal models paired with behavioural tasks. Furthermore, mouse models relevant to dementia are essential for the understanding of the progressive changes in vascular and neuronal functions leading to cognitive decline.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Our estimated number of animals required for this project is based upon our published work and preliminary data, and more broadly to my 15-year experience in using mice for brain research. This number reflects an estimate of combined cross-sectional and longitudinal cohorts covering all the proposed experiments involving animals, which also includes breeding and maintenance of lines.

Importantly, I have extensive experience in managing multiple mouse colonies from maintenance/breeding purposes up to the actual mouse procedure/euthanasia. Also, I have experience in writing successful grants and original articles where experimental design was involved.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In order to ensure we are sufficiently powered to answer the questions posed while still adhering to the principles of the 3Rs for animal use, we have used the Experimental Design Assistant (EDA) tool from the NC3Rs and also estimated sample sizes for each experiment using effect sizes from previously published work or preliminary data. To further reduce animal use, longitudinal cohorts will be favoured over cross-sectional studies. Finally, the same cohort of animals will undergo different imaging procedures, as well as behaviour testing, without causing greater harm to those animals.

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 in vitro systems will significantly reduce the number of animals as comparison of control and treatments can be generated using a single animal, while in vivo studies would require one animal per condition. Moreover, brain tissues will be shared among researchers to reduce overall mouse usage. Also, pilot experiments will be performed to optimize an imaging protocol for instance, which will help to optimise the total number of animals used. Finally, efficient breeding strategy will be applied in order to obtain the right genotype and the right littermate controls with a minimum amount of crossings.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Manipulation of brain vascular cells can only be meaningfully studied in the mammalian brain, as they rely on specific cell interactions that are temporally and microenvironmentally restricted. To date, experimental systems that allow this are limited, and must involve vertebrate animals such as mice.

This is particularly true with respect to the proposed experiments, which will involve the physiological and genetic assessments of vascular cells and functional measures of blood-brain barrier in relation to brain/cognitive functions. We will use cutting-edge neuroimaging methods, such as magnetic resonance imaging, positron emission tomography, and two-photon laser microscopy, to image the living rodent brains under anaesthesia. Gentle handling in combination with proper induction and maintenance of general anaesthesia will mitigate the potential pain, suffering or distress during and after imaging sessions. All animals are expected to make a rapid and unremarkable recovery from the anaesthetic within one hour. Uncommonly animals that fail to do so or exhibit signs of pain, distress or of significant ill health will be humanely euthanised. In addition, we will also perform behavioural assessments in parallel.

Why can’t you use animals that are less sentient?

Studying vascular function and dysfunction in vivo and relating these findings to cognitive performance is simply not feasible with less sentient animals (e.g., flies or zebrafish). The

use of cutting-edge magnetic resonance imaging techniques in flies and fishes is not possible to date. Also, rodents more closely model human physiology than flies or fishes. Non-sentient alternatives are not available to address the questions posed in this project as those beings do not possess a nervous system which is centralized (i.e., starfish) or do not have a nervous system at all (i.e., sponges). Finally, using a more immature life stage won't be relevant as we are studying ageing and dementia, and performing terminal anaesthesia is not feasible as the goal is to follow animals longitudinally and possibly link early vascular dysfunction to downstream consequences, i.e., neuronal and cognitive dysfunctions.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

As we move forward with our first/preliminary experiments, we will gain greater knowledge on the pathophysiological events that are temporally and spatially restricted. These important data will allow us to adjust our imaging protocols for instance, and possibly reduce the number of scans per animal as well as shorten scanning time. All the animals undergoing an imaging session and/or any treatment will be closely monitored to detect signs of distress like piloerection (i.e., goose bumps), weight loss, abnormal locomotion, or hunched posture.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the guidelines produced by the NC3Rs (https://nc3rs.org.uk/guidelines) to ensure experiments are conducted in the most refined way.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will regularly pay a particular attention to the outcomes of both 3Rs and Animal Welfare committee at a local level as they meet four times a year. Also, we will attend the annual 3Rs day to stay up to date with the advances in the 3Rs, particularly refinements, within our establishment and beyond.

These events will allow us to, not only stay inform about the latest advances in animal welfare, but also adjust our animal protocols accordingly on a yearly basis if not more.

63. Targeted mouse models for pre-clinical studies in paediatric 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

Childhood Cancer, Refined therapy, Genetically Engineered Murine Models (GEMMs), Chemo- resistance, Patient derived xenografts (PDX)

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 use mouse models for preclinical studies aimed at refining treatments for children with high-risk paediatric cancers.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

A significant proportion of children with neuroblastoma (an abdominal tumour) or medulloblastoma (the most common malignant paediatric brain tumour) are considered high risk. Despite intensive treatment they have a very poor prognosis and experience both resistance to therapy and widespread tumour metastasis. The events responsible for this are unknown. In addition, many of those children that do survive suffer a number of late effects that are severe or life limiting. There is a definite need to improve treatment options for these children.

What outputs do you think you will see at the end of this project?

Publishable data as to how tumours become resistant to therapy. Presentations of data at International meetings, collaborations with clinical, academic and pharmaceutical partners to further interrogate biological unknowns.

Mouse models and pre-clinical platforms to be used by others in this field of study.

Publishable development of pre-clinical therapeutic strategies to treat resistant tumours, potentially leading to clinical trials in patients.

Who or what will benefit from these outputs, and how?

In the short term we will generate novel in vivo models that better mimic the genetic landscape of human disease and gain more insight into the mechanisms behind metastasis and treatment resistance in these tumours. We will use these to pre-clinically test standard treatments and compounds obtained through our collaborations with external researchers or organisations. This data will also be of use to the wider scientific community, pharma companies and the clinical profession.

Our medium-term aims are that by characterisation and analysis of newly developed models and further development of our existing models we will be able to target the relevant mechanisms and suggest new therapeutic approaches in order to carry out pre-clinical trials with monitoring of markers to measure response. This will allow us to strengthen our relationship with industry as well as the academic and clinical community.

In the long term we will produce data leading to clinical trials. We hope to develop therapeutics that have greater specificity and hopefully fewer side effects this will be of huge use to industry, the clinical community but ultimately to patients.

How will you look to maximise the outputs of this work?

This work will be part of a national network with expertise in mouse genetics initiative, and as such this will be a great forum for collaboration and sharing ideas, and technologies. We aim to disseminate our findings through publication in peer reviewed journals and by presenting data at relevant conferences.

Species and numbers of animals expected to be used

- Mice: 15000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use genetically engineered mice that are pre-disposed to develop tumours post-weaning, these represent the high-risk disease seen in children. These are the lowest sentient species that are appropriate as they faithfully recapitulate the complex physiology

which leads to the development of this type of disease and have comparable metabolism and immune responses.

Typically, what will be done to an animal used in your project?

We will breed mice that are genetically engineered to develop tumours. These may be neuroblastoma tumours which arise in the abdominal cavity or medulloblastomas which arise in the brain. We will inject some mice with tumour cells either from patients or mice and observe these for tumour development. Mice will be assessed for tumour formation using the most sensitive techniques to detect tumours as early as possible. These mice may undergo non-invasive imaging to follow the tumour progression. These mice may be given multiple drug treatments over a number of weeks that mimic the type of approach used to treat these tumours in patients. The tumours will initially shrink in response to treatment, but when the tumours return they will be killed and the tumours taken for studies as to why they have relapsed with the aim of understanding what happens in patients. In some mice we may obtain tissue and blood for biomarker monitoring.

What are the expected impacts and/or adverse effects for the animals during your project?

Neuroblastoma tumours normally arise in the abdominal cavity and occasionally in the thoracic cavity, the primary adverse effect is weight loss, if the animals lose 15-20% body weight that doesn't respond to diet supplements they are humanely killed. The primary symptoms of medulloblastoma, a tumour in the back of the brain, are either circling behaviour or domed heads, when they show these signs they are humanely killed, where possible we use non-invasive imaging to detect tumours before the onset of these symptoms. Using our existing models, we have a great deal of knowledge as to the expected symptoms and time of onset and thus are able to prevent progression of tumours to the stage that they cause ill health. With our new models we will monitor the health of the animals carefully and where possible use non-invasive imaging techniques prior to onset of symptoms. We will test methods of treatments such as surgery and drugs. This could cause pain and weight loss. Pain relief and diet supplement will be given and if they don't respond 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)?

For our genetically engineered models we expect 30 - 40 % of animals to develop a tumour. We expect 60-70% to be non-tumour bearing and hence will have a sub-threshold severity. The majority of tumour bearing animals will be placed on a pre-clinical trial and may be given either a vehicle control - whereby the tumour will continue to grow or given the experimental compound, where the outcome depends upon the efficacy and the type of trial undertaken. The expected severity for most of these will be moderate (25-30%) or mild (5-10%). For our implanted tumour models we expect 50 - 70 % to develop tumours, depending upon the cells/tumour type implanted, these may be used in pre-clinical trials or may be used for tumour passage, these would have 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 development of tumours depends upon a number of interactions between the tumour cell and the local tissue environment. Tumours can actually be classified as organs as they comprise a multi-tissue organisation and are continually responding to different environments and stimuli. They are comprised of cellular, structural, and molecular components collectively known as the tumour microenvironment (TME), which hosts a heterogeneous cellular population of cancerous and noncancerous cells such as fibroblasts, stromal cells, and immune cells, which migrate from the bloodstream and neighbouring tissues.

There are many studies aiming to differentiate the relative contributions of these structural, molecular, and microenvironmental processes in disease progression in neuroblastoma. There are also many attempts to use tissue engineering strategies to model neuroblastoma and medulloblastoma in vitro,such as the multicellular tumour spheroid (MCS) models, scaffold-based platforms and hydrogels. Whereas these 3D cancer models offer many advantages over 2D cultures, and they more closely reflect the in vivo tissue organisation, they are limited in the ability to simulate in vivo tissue conditions at the organ level; as they lack other tissue properties such as a developed vasculature, perfusion, and other cell-cell interactions. They represent short-term or static conditions, in contrast to the continually progressing in vivo system.

There have been models of both neuroblastoma and medulloblastoma developed in zebrafish resulting in tumour histology comparable to their human counterparts. There is, however, a disparity between zebrafish physiological temperature (28/29°C) and human body temperature (37°C) which is likely to affect many cellular processes including enzymatic and metabolic activity, both fundamental in therapeutic response. For therapeutic studies there is also the complication of administration via water which may not be suitable and the absence of general guidelines to calculate mammalian equivalent doses from zebrafish doses.

There is also a chick chorioallantoic membrane (CAM) model of neuroblastoma which has been used for investigation of tumour differentiation, proliferation, invasion, and migration. This model has the disadvantage of a lack of immune system and that avian drug metabolism is different from humans.

Medulloblastoma has the additional complication that it is in the brain and any therapeutic compound has to be able to penetrate the blood brain barrier - this is difficult to model in other systems.

Which non-animal alternatives did you consider for use in this project?

Currently our work flow involves selecting compounds that have either shown efficacy in another type of cancer, or have been developed to target an aberration seen in these paediatric tumours. We assess them for efficacy against their target in 2D cell culture systems before proceeding to in-vivo trials. We have considered developing multicellular tumour spheroid (MCS) models from patient derived tumour samples in order to triage our

compounds before they are tested in mice. The availability of material is not consistent, biopsy samples are small and priority for these is obviously for diagnostic testing not research. Resected tumours are often unsuitable material for culture as they are usually taken after the patient has chemotherapy and therefore there is a lot of dead tissue and hence it makes it hard to establish cultures.

We are also trying to develop patient derived xenograft models, where, although these still use mice they could lead to a reduction of mice needed for trials if reproducible reliable cohorts of animals were available harbouring identical tumours, avoiding the need to breed animals some of which are non- tumour bearing. However, this work is hampered by the availability and suitability of the source material.

Why were they not suitable?

Although these models are useful for initial screening of anticancer compounds and for extrapolating the biology and characteristics of neuroblastoma and medulloblastoma. None of the discussed models can completely replicate the complexity of human cancer due to the interspecies variations in organismal organisation, tissue architecture and composition, and immune systems all features that are important in therapeutic testing.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Prior knowledge of our genetically engineered murine models allows us to predict how many mice to breed to obtain numbers for our experiments. We will optimise our breeding strategies using the expertise available at a national facility and have based calculations of numbers on these strategies. We have used data from our previous experience running pre-clinical trials in the statistical analysis to inform us as to how many mice are required for individual experiments.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have a workflow whereby we test the validity and relevance of each new model we develop and therefore make decisions as to how to proceed with minimal number of animals. This depends on the penetrance and variability of the model. For those models with highest penetrance we can vastly decrease the size of animal cohorts used in either biologic or preclinical studies. For evaluation of interventions including drugs/biotherapeutics we determine a minimally effective biologic dose in pilot experiments and then test in optimised cohorts. We will refer to tools such as the NC3R's Experimental Design Assistant and local statistical expertise to assist. Experiments will be designed to use the minimum number of mice whilst providing statistically and biologically significant results. We use detailed detection modalities and imaging to minimise use, selecting only those animals that form tumours, very early, and then streamlining the duration of their

treatment. In our experiments we use both sexes to optimise the use of mice of the desired genotype.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

When breeding our genetically engineered mouse models we will design breeding programmes that are optimised to deliver maximal tumour penetrant density in each colony, and as above, we optimise cohort size using biologic and imaging endpoints. We use SOPs to standardise the way experiments are run and data collected in order to reduce variability and sample size. We use biomarkers to determine response which permits a rolling evaluation design in experiments designed to detect therapeutic effect or survival extension. We are working as part of a national network with expertise in mouse genetics, this is an ideal opportunity to share both information and resources, potentially including data, models or samples.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will be using genetically engineered mouse models of neuroblastoma and medulloblastoma. We will use models that faithfully represent the disease as it presents in patients. We have previously demonstrated that we are able to produce and utilise our models to advance clinical trials. We are advancing our previous research as there is increasing data as to the importance of the immune system in cancer development and in response to therapy. We are able to interrogate the immune response in tumours using spatial phenotyping technology and make important comparisons between the mouse models and human cancers. Depending upon the type of study we will define the humane endpoint based on our prior experience but these are all within the moderate severity rating and intervention occurs at the earliest point possible that will give meaningful results.

Why can’t you use animals that are less sentient?

There is not currently any other organismal models of cancer with the appropriate metabolism and immune system in which to under take this type of research. Zebrafish have a lower physiological temperature making them unsuitable for therapeutic intervention trials. We use models that are very representative of the clinical disease. The location of tumours is relevant to the treatment plans, for example a major consideration as to treatments for our brain tumour models is whether the drugs are able to cross the blood brain barrier, thus testing in a model that addresses this issue is crucial. We need to assess the effects of drug treatment on living animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Using information from our current models as to potential symptoms we will closely monitor all animals that are expected to develop tumours. Any new drug regimens will be discussed with Named Persons, i.e. the vet and animal care and welfare officers, in advance. We will use refined techniques such as home cage monitoring, regular observations, combining drugs to reduce the number of injections and use of minimally effective biologic dose with pharmacodynamic confirmation, and optimised pharmacokinetic dosing, to minimise harms. Imaging and biomarker based tumour detection will also be employed to minimise uncontrolled growth of tumours and symptoms. We will use analgesia and anaesthesia as recommended to minimise any pain from surgery. There will be staff training across both institutions used in this study who will regularly meet to discuss refinements in animal care.

When animals start to show any of the specific symptoms relating to pain from the tumours they carry or the procedure they are undergoing, they may be given analgesia if the application of such agents does not affect the experimental outcome.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the ARRIVE guidelines 2.0. For surgical procedures we follow the LASA guidelines on aseptic procedures (LASA 2017 - Guiding Principles for Preparing for and Undertaking Aseptic Surgery).

The animal facility has full AAALAC and ISO9001-2015 accreditation. To conform to these standards, we must work to a high level of quality control on all fronts including husbandry, phenotyping and administrative processes.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Through our collaborative network we are exposed to data from other model organisms and we will follow developments in alternative technologies, especially at conferences and in publications. We receive regular updates from our animal care teams and AWERB; we will share refinement and animal care knowledge, specifically of cancer models, with all involved in this project .

Staff at the facility we are using adhere to the highest welfare standards and are proactively committed to implementing the 3Rs for the benefit of mouse health and welfare. Staff regularly attend animal care meetings and keep up-to-date with the current guidelines.

64. Antibody Production

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Antibody production, Polyclonal antisera, Monoclonal antibody

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 Licence is to provide a resource for the production of high quality, highly specific and sensitive non-commercially available polyclonal and monoclonal antibodies for use in studies across a broad range of scientific disciplines. These projects range from basic research to applied clinical translation.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Antibodies are made by the body as a defence mechanism against foreign material, such as bacteria and viruses. They circulate throughout the blood and lymph and attach themselves to this foreign material. In this way they help to protect animals and humans from disease by stimulating an immune response.

Scientists can make use of this natural ability by challenging animals with foreign material causing the animals to create very specific antibodies to this material which are then isolated from these animals. This has revolutionised biomedical research, providing

scientists with highly specific and sensitive clinical reagents for investigation.

It is important that these important scientific tools can be raised in a competent and ethical manner. This Licence aims to provide a generic ‘service’ Licence under which the scientific community can create antibodies that are not available commercially without the need to raise a large number of individual Licences.

Whilst techniques exist to produce similar reagents without the use of animals they are not yet universally applicable, and traditional animal-based alternatives remain required. This License will ensure robust, legitimate scientific justification exists for the ongoing use of animals in antibody production.

What outputs do you think you will see at the end of this project?

Given the breadth of studies likely to be undertaken using this Licence, this ensures the outputs of this Licence will be both varied and many. These range from the advancement of a scientific hypothesis, information dissemination (through peer reviewed research paper publication or public presentation) to potential product creation, in the form of diagnostic assay development or route to clinical therapy.

Who or what will benefit from these outputs, and how?

The antibodies produced under this Licence have a wide range of potential applications ranging from furthering basic understanding of biological processes, through to offering bespoke reagents for use in disease diagnosis and/or therapy. This breadth of applications ensures short-term (immediate research), medium-term (diagnostic application) and longer-term (clinical need) benefits are observed and will ensure the maximum number of people (scientists, companies, the public) see a benefit from this work.

The types of antibodies that scientists require are highly variable dependent upon the area of research. Such antibodies are rarely available from commercial suppliers.

It is important that scientific investigators have access to a method of producing individually tailored antibodies to a target of interest. As antibodies are so commonly used in research there is also a need for them to be made efficiently and in a way that is least harmful to the animals involved.

How will you look to maximise the outputs of this work?

All data produced in a particular project plan is shared with the Customer to promote full collaboration and data transparency. This includes all negative data so that an approach may be improved or refined to increase the likelihood of future success. As part of this process, all information related to animal welfare is also shared so that any potential improvements may be made accordingly, not only for a particular project, but so may be applied to other projects carried out under this Licence.

We propose introducing not only retrospective assessment of the licence, but also regular, annual assessment of the licence between the PPL, PIL, AWERB, NACWO, Veterinary and 3R's Committee within the Establishment. This way, learnings (scientific, welfare, 3R's etc) can be promoted and disseminated to further improve the outputs of this work.

Species and numbers of animals expected to be used

- Mice: 840
- Rats: 50

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

In this Licence we will be using laboratory animals only from approved commercial providers. Typically these animals are BALB/c mice and Wistar rats. Both species have a well established history in the field of antibody production due to the generation and availability of plasmacytoma cell lines allowing monoclonal antibody production from these strains. The genetics of these animals is also well studied and characterised extensively, making them the most robust and suitable strains for this work. All animals used will be immunologically mature (over 8-12 weeks old).

Although it is *possible* to produce antibodies *in vitro*, such techniques are not sufficiently precise, reliable or economically viable to replace existing *in vivo* methodologies. Despite over 40 years of additional research and development into alternatives, techniques such as phage display, ribosome display and de novo engineering simply do not provide a robust, reliable or readily available alternative to using traditional *in vivo* techniques, which to this day remain the 'gold-standard' for the vast majority of research, development and therapeutic applications.

Typically, what will be done to an animal used in your project?

Animals (typically mice or rats) will be immunised using a range of reagents (termed 'antigens') that are designed to stimulate an immune response in the animals, subsequently leading to the production of antibodies and antibody-producing cells in the host animal. These antibodies or antibody producing cells can then be harvested from the animal by way of blood or tissue removal and analysed for specific use. Animals typically remain on procedure for around 24 weeks and are subjected to up to 8 immunisations / harvesting procedures during this time. Animals may experience transient pain but no lasting harm from administration of substances by injection or blood sampling procedures.

What are the expected impacts and/or adverse effects for the animals during your project?

Whilst on procedure, animals (typically mice or rats) may experience transient pain but no lasting harm from administration of substances by injection or blood sampling procedures. They recover quickly and can be given painkillers and post-procedure care 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)?

Mice: >95% Mild, <5% Moderate

Rats: >95% Mild, <5% Moderate

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Although it is *possible* to produce antibodies *in vitro*, such techniques are not sufficiently precise, reliable or economically viable to fully replace existing *in vivo* methodologies. Despite over 40 years of additional research and development into alternatives, techniques such as phage display, ribosome display and de novo engineering simply do not provide a robust, reliable or readily available alternative to using traditional *in vivo* techniques, which to this day remain the 'gold-standard' for the vast majority of research, development and therapeutic applications.

However, we are mindful of the guidance in the Antibody Report by the Project Licence Strategic Review Subgroup (2022) and the EURL ECVAM (2022) report suggesting that non-animal based methods for antibody production are becoming a potentially viable alternative to traditional methods of antibody production.

We fully appreciate and agree that the use of animals should not be the 'default' position. Any use of animals should be fully justified by robust, legitimate scientific justification, as explained in the 'Action Plan' section of this application.

Which non-animal alternatives did you consider for use in this project?

As above, whilst 'synthetic' approaches are available (phage display, yeast display, ribosome display etc), they are not yet fully suitable alternatives to established *in vivo* technologies. The reasoning for this is explained in detail, below.

However, as a business keen to keep up with current scientific practice, we have already committed funds and resource into exploring the production of antibodies in-vitro, thus potentially avoiding the use of animals in the future. This is a 3-4 year plan to develop the scientific technology to not only match current providers, but to overcome the many real obstacles associated with in-vitro antibody production.

Why were they not suitable?

Many of the synthetic approaches listed above (phage display etc) are not yet established enough to provide a robust, reliable, easily accessible and non-restrictive platform (scientifically, economically or commercially) for routine antibody development. Reasons for promoting and continuing existing hybridoma technology over newer, alternative technologies can be broken down in to the following categories:
Scientific Rationale:

Alternative display technologies are not routinely available to researchers without a significant cost and scientific expertise barrier (see 'Commercial Rationale' below).

Many (particularly academic) researchers simply need a very small quantity of relatively crude (non-purified) material for confirmation of a hypothesis or for inclusion into a research publication. This would not be scientifically or economically possible using alternative technologies, given the reasons outlined below.

The rapid speed of which we can currently operate provides a scientific and competitive advantage to users of our existing licence in the provision of antibody-based materials. During the CoVID19 pandemic, we were able to rapidly supply antibodies to CoVID19 proteins furthering the research into viral transmission and dissemination.
The products typically generated by alternative technologies are not complete antibodies, but instead antibody 'fragments' (single chain antibodies, single domain antibodies etc) and hence need to be 're-engineered' into a more conventional format for use in many subsequent assays. This requires time, cost and significant optimisation of expression.

Expression of antibody fragments is typically performed in a 'transient' manner. This means that only small batches of material can be produced at any one time (due to to production cost, operational and logistics constraints), meaning the process is not easily scale-able when larger or repeat quantities are required.

Antibody fragments are often monovalent and as such may have a lower affinity for their respective antigen as compared to larger, divalent whole antibody molecules.

Antibody fragments are often produced using a combinatorial 'library' approach. In this approach, DNA libraries are created that may correspond to naturally occurring antibodies. These libraries are often termed 'naive' libraries, as they are not generated from individuals with or with previous exposure to a specific antigenic target. This often results in antibodies being produced which may be of lower affinity and specificity than compared to antibodies produced when animals are immunised directly with the same antigen of interest.

As part of the library selection process antibody binding to antigen is determined using a process called 'panning'. This process relies on the production of highly purified antigen, free from cellular or protein contaminants. Unfortunately, in many instances this is not available, which significantly impacts the panning process and subsequent selection of antibodies binding a range of protein components. This is particularly true of cell-membrane associated antigens (such as G protein coupled receptors - the largest class of drug targets currently in testing), which cannot be easily expressed recombinantly and as such antibodies cannot be easily raised using these library based methods.

If access to traditional, well-established (and highly successful) technologies are restricted in the UK, this is likely to lead researchers to search for non-UK based service providers. This may lead to a lack of standardisation of research processes (lack of ISO quality standard implementation, for example) and subsequent differences in results generated caused by this.

Commercial Rationale:

As the majority of phage display libraries are held by large commercial providers, this poses a significant cost barrier to the vast majority of academic and SME-based

researchers who need access to low-cost, readily available and highly-skilled UK based service provision.

As many of these alternative technology commercial providers reside outside the UK (in terms of manufacturing and physical service provision), this presents a risk to UK-based business and the loss of creation / maintenance of scientific IP within the UK.

The overwhelming majority of users of our current Antibody Production Licence are UK academics and SME's who rely on a rapid low-cost and intellectual property free production service from skilled UK based researchers, which ensures ease of communication and transparency of results and information which can be freely shared given the publicly available technology details.

Cost for monoclonal antibody production is typically ten fold higher when using commercially available alternative technology service providers compared to current hybridoma approaches.

Cost for polyclonal antibody production is typically twenty fold higher when using commercially available alternative technology service providers compared to current hybridoma approaches.

Even when alternative technologies are 'in-sourced' into academic laboratories, the overall set-up and use cost (even excluding skilled scientific support time) is typically five fold higher than compared to conventional antibody approaches.

Time to generate required antibodies is significantly extended using alternative technologies.

As alternative production technologies are typically the proprietary products of commercial providers, specific details with regards production methodology may not be shared with the end- user thus restricting freedom of information and scientific/commercial progress. In addition, the time and expertise required to review legal agreements with the commercial provider adds a significant time delay and cost expense to project inception.

Furthermore, due to this proprietary nature, commercial providers retain significant intellectual property rights over projects and products generated from use of their technologies. This prevents dissemination of information and products generated and restricts subsequent commercial freedom to use these products in diagnostic or clinical applications.

In addition, this allows commercial entities to significantly benefit (for the long term) from charity or grant-funded research.

Those commercial providers that utilise alternative display technologies almost always also offer conventional polyclonal and monoclonal antibody generation indicating the maintained scientific and societal need for these reagents.

During the CoVID19 pandemic our facility did not close for one day and all ongoing projects were maintained, resulting in valuable CoVID19 related work being undertaken. This was not the case in many commercial providers which resulted in a delay in advancement of CoVID related research.

UK Economic Rationale:

If access to traditional, well-established and highly successful technologies are restricted in the UK, this is likely to lead researchers to search for non-UK based service providers. This may a lead to a loss of funding to the UK economy, a decrease in the use of UK businesses, a subsequent increase in carbon-footprint due to increased shipping and logistics and a reliance on oversees support.

Animal Welfare Rationale:

If access to traditional, well-established and highly successful technologies are restricted in the UK, this is likely to lead researchers to search for non-UK based service providers. In the current scientific landscape, this is likely to be in China or Eastern Europe where animal welfare standards do not meet current UK provision, thus potentially resulting in greater animal use, suffering or cruelty.

Summary:

To conclude with regards alternative display technologies, whilst they may have some merit in certain scientific applications (for example where antibodies to conserved mouse proteins are required) these applications are likely limited to a select number of projects and hence are not suitable for the vast majority of users of a service Licence intended at providing basic research tools and needs.

In summary, it is essential to UK science that researchers have a pathway to access established and successful hybridoma technology for generating high affinity, highly specific, whole antibody reagents in a manner which is scientifically, commercially and economically viable whilst at the same time promoting the highest standards of welfare and best practice within the animal care community.

We believe all items listed above fulfill the criteria listed in the Antibody Report by the Strategic Review Subgroup and commit towards further continual improvements to the 'Replacement' category.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 significant over-estimates based on the number of animals used in our current Antibody Production Licence. Over the 5 year period, these numbers are:

Mice: ~400 Rats: 0

The increase in estimated numbers from current is in line with expected business increase due to enhanced sales and marketing activities.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Over the past twenty five years we have gained considerable expertise in the use of animals for antibody production.We continually review animal usage with a view to minimisation, and generally no more than two rats or 4 mice are used for the production of each antibody, allowing for variation, unexpected mortality etc.

However, in line with the Antibody Report from the Strategic Review Subgroup (2022), animal use is predicated on the lack of non-animal alternatives being available.

Past studies have seen a single animal immunised with multiple antigens, with the aim of reducing the overall number of animals used. This has shown differing results based on the antigen, with animals immunised with very closely related antigens (for example, with small amino acid variances in immunogen sequence) showing a lack of clear specificity when the serum response is screened against the same antigens independently.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Our standard antibody production process uses 4 mice, which is the minimal number of animals required based on the heterogeneity of a typical immune response. This has been refined over many years to ensure the maximal scientific output is achieved with the minimum animal usage. Our standard response to Customer enquiries regarding use of rats is to only use 1 animal unless necessary to use additional.

We believe the items listed above fulfill the criteria listed in the Antibody Report by the Strategic Review Subgroup (2022) and commit towards further continual improvements to the 'Reduction' category.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Throughout this study we will use animals purchased from commercially licensed breeders and using the smallest number of immunologically-mature animals possible per study group. At all times, minimum severity limits will be adhered to. As we have successfully refined this process over 25 years, this ensures the maximum scientific output whilst maintaining the highest levels of animal welfare by reducing study times, animal numbers, and animal suffering etc. by continually reviewing best practice. Specifically over the duration of our current project licence we have introduced the use of non-aversive animal handling techniques. Animals are never lifted by their tails, only ever by 'cupping' or using approved handling tunnels. Furthermore, recently we have introduced the use of 'Vetbeds' into our sampling process. This allows animals free reign to move whilst the procedure is

undertaken. Once the procedure is complete, the animals are also then provided a small food treat as a high value reward.

During this project animals will be immunised using methods similar to human vaccination (e.g. injection of substances under the skin, into the peritoneal cavity or by intravenous injection) for stimulation of the immune response to make antibodies for further study. As part of ongoing personal and professional development and NC3R's processes, we commit to actively searching for ongoing refinements and improvements with regard animal welfare procedures and standards over the course of this license.

Why can’t you use animals that are less sentient?

Antibody production relies on the study animals having a fully developed immune system. We cannot use embryos or very young animals as their immune system is immature and does not respond to antigenic stimulation in the correct way that will harness a diverse antibody response. Animals typically begin procedure aged 10-14 weeks and remain on procedure for around 24 weeks until study completion

Use of non-mammalian animals are limited as they do not produce a classical 'antibody-based' response to antigenic challenge and so do not provide a suitable end-product for further study.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The protocols and practices proposed in this Licence are based on over twenty five years experience of producing antibodies and reflect the widely available literature regarding appropriate strategies for antibody production. This typically results in the production of high affinity antibodies in the shortest possible timeframe and with the least amount of suffering to the animals used.

As part of ongoing personal and professional development and 3R's processes, we commit to actively searching for ongoing refinements and improvements with regard animal welfare procedures and standards over the course of this license.

As a further review point, we suggest introducing regular formal review meetings with the local AWERB to both retrospectively assess licence activity, suitability and efficacy, but also to review any wider learnings, outcomes or opportunities for continuous improvement identified throughout the course of the license. This is in addition to the regular interim reviews carried out by the 3R's committee (a sub- committee of AWERB) at the named Establishment to ensure License compliance and seek process improvements where required.

Whilst not initially submitted, a further future option is to consider adding a specific protocol (in the form of a project amendment) which allows for exploratory or development work in assessing new 3R's approaches that may also improve scientific output.

Furthermore, as a business keen to keep up with current scientific practice, we have already committed funds and resource into exploring the production of antibodies in-vitro, thus potentially avoiding the use of animals in the future. This is a 3-4 year plan to develop the scientific technology to not only match current providers, but to overcome the many real obstacles associated with in-vitro antibody production.

All work is carried out to the ISO 9001 quality standard which requires full documentation and auditability for all aspects of the work, thus refining the processes involved and ensures consistency across all projects.

We regularly review animal usage with a view to reduction and the use of alternatives. Animal suffering is reduced by working to mild/moderate severity limits and ensuring staff are well-trained in the handling, sampling and husbandry of all animals. Additional measures include the use of low volumes of antigen, injection of well-prepared/emulsified antigens by fine-gauge needles and the split injection volumes across multiple sites to avoid local tissue reaction. Other measures include non-aversive animal handling techniques, removing use of animal restraining devices, and 'reward-based' training of animals. Post procedure all animals are placed into a supportive environment (head-pad/incubator with access to high energy food) and are regularly monitored to ensure timely action is taken in the event of ill effect.

After a previous comparative test of adjuvants, we still find Freunds to be the most reliable in terms of performance, however, we will continue to test different adjuvants as they become available, and will actively encourage potential Customers to consider alternatives to Freunds adjuvant where no previous data supporting the use of Freunds adjuvant exists. Where possible, studies may be performed to assess suitability of different adjuvants to limit potential issues of ill effects caused by specific adjuvant use (See 'Project Plan' section).

Animals do not normally suffer significant ill effects from the procedure and the use of analgesics or anaesthesia is not usually necessary apart from the terminal bleed. Prior to study inception, all immunising agents are assessed to determine potential for ill-effect (for example, use of toxins, chemical composition, pH, likely inflammatory effect etc.), thus minimising potential for harm during the immunisation process. We will obtain veterinary advice for animals which show any sign of distress of discomfort. Animals showing significant signs of distress will be culled by Schedule 1 methods.

We believe all items listed above fulfill the criteria listed in the Antibody Report by the Strategic Review Subgroup and commit towards further continual improvements to the 'Refinement' category.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Animal Welfare Best Practice:

Refining procedures for the administration of substances (https://journals.sagepub.com/doi/pdf/10.1258/0023677011911345)

A guide to defining and implementing protocols for the welfare assessment of laboratory animals (https://journals.sagepub.com/doi/full/10.1258/la.2010.010031)

Scientific Best Practice:

Harlow E. and Lane D. (1988) Antibodies. A Laboratory Manual. CSH Press (This text remains Gold- Standard for antibody production)

Monoclonal Antibody Production (1999). Committee on methods for producing monoclonal antibodies (https://www.ncbi.nlm.nih.gov/books/NBK100199/pdf/Bookshelf_NBK100199.pdf)

Our own Standard Operating Procedures (2023) ISO:9001 Approved.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Working closely with a variety of clients and our in-house teams allows us to keep up to date with all developments in relation to the 3R's, the latest in animal welfare developments and Licence reporting requirements.

In addition, our experienced scientists regularly review the published literature for scientific and 3R's advances that may positively impact work carried out under this Licence with a view to potential discussion and implementation into future study requests. This includes includes reviewing the NC3R's website and receiving newsletter updates.

65. Breeding and maintenance of genetically altered strains with validation and refinement of techniques used in archiving, rederivation and creating genetically altered 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

Cryopreservation, Reduction, Refinement, Genetic Alteration

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 support research into increasing the human health span by producing genetically modified animals as a resource for all our researchers. We apply the 3Rs principles of Reduction, Refinement and Replacement to ensure that the smallest number of animals suffer the least amount of harm to allow us to reach research goals.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Although medical advances have increase human lifespan, quality of life is often reduced by age associated diseases including diabetes, Parkinson's, Alzheimer's and many others.

The research here looks at increasing how long people can stay healthy.

This project provides core services in support of the research on site by:

Establishment and maintenance of high health status colonies of Genetically Altered (GA) and wild type mice without genetic drift. Genetic drift is the change in frequency of particular genes in small populations over time due to individuals failing to reproduce. Central management of core colonies reduces excess breeding and allows production of large, age matched cohorts. This allows researchers to design experiments that can test several variables at once with one control group, instead of sequential experiments which each require a control group. This reduces the number of mice used in experiments.

Maintaining a bank of sterile male mice as a service for all users. Females used as recipients for GA or wildtype embryos, will not carry pups to term unless they have been mated. By pairing them with sterile males they become pseudo-pregnant, that means that embryos transferred into them will implant, but because their own eggs have not been fertilised they will not compete with the transferred embryos.
Sterile males will be produced either by surgical vasectomy or by breeding a GA colony, the mice have no ill effects from the genetic modification, but approximately 50% of the males are sterile. We can tell which ones they are because the alteration is also linked to coat colour so there is no need to take tissue samples to genotype them. The fertility of the females is not affected.

Other Benefits:

Freezing sperm and embryos of GA and wildtype strains provides a contingency in the event of damage or disease in the animal holding units.

Creation of new lines of genetically altered mice in support of already approved research projects

Quality control of reagents, equipment and protocols used in the other objectives. And quality control of frozen stocks of embryos and sperm.

This project will investigate reports of new or improved methods, technology, equipment and strains that could increase the effectiveness or scientific potential of our current methods to achieve our other aims. We will test the validity, perform a cost benefit analysis and incorporate into our procedures those that will, on balance improve the welfare of the animals we use.

What outputs do you think you will see at the end of this project?

We will establish and maintain high health status wild type, immunodeficient mice and sterile male mice as a service for other approved projects. We will test equipment, procedures, alternative strains of mice and make quality-controlled reagents and media used in:

- production of sterile male mice embryo production and culture
- freezing and thawing of sperm and embryos revival of lines from frozen embryos and sperm IVF
- creation of new lines of genetically altered mice

We will also perform quality control tests on the frozen sperm and embryos

Who or what will benefit from these outputs, and how?

This project will reduce the number of mice needed for experiment by maintaining high health status production colonies free from undesirable genetic traits. This is important as the outcomes of experiments are more consistent if the subjects are all similar and this in turn increases the statistical significance of the results.

We will reduce the numbers of genetically modified animals kept alive and breeding through cryopreservation.

This project will also deliver a reduction in numbers of breeding animals needed by using in-vitro techniques for rapid colony expansion.

There is potential for reduction and refinement site-wide if new techniques for embryo production, cryopreservation, embryo transfer or creation of new genetically altered lines are demonstrated to be a refinement or more efficient than our current methods.

Improvements in animal welfare are achieved by being able to import or export lines as frozen sperm or embryos thereby avoiding stress caused by live animal transport.

This is of potential benefit to other researchers and collaborators world-wide as we can send them frozen stock.

Potentially animal welfare could be improved by refining techniques.

Other projects or establishments that use genetically altered mice, but lack the facilities or expertise to manipulate embryos, freeze or thaw embryos and sperm or perform embryo transfers may benefit from the services of skilled technicians here.

How will you look to maximise the outputs of this work?

Our scientists often need large, age and/or sex matched cohorts to test several different variables. We will maximise the impact of our work by keeping the numbers of genetically modified animals bred to the smallest numbers consistent with producing the animals required.

Species and numbers of animals expected to be used

- Mice: 17,720

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use mouse eggs, sperm, embryos, juveniles and adults. Our scientists overwhelmingly use mice as models and our work provides these models for their use.

Typically, what will be done to an animal used in your project?

By far most of the animals used on this project will be used in breeding and maintenance of genetically modified mice. The majority of these will be sub-threshold.

Immunodeficient strains of mice are bred and maintained in a high health status unit. Protocols in place for husbandry ensure that the mice are unlikely to catch any diseases and therefore do not suffer adverse effects from their genetic alteration. Very few of the mice if any will require ear biopsies for genotyping.

Superovulation generally consists of two (occasionally up to four) injections of hormones and or other substances (e.g. antibodies) that have been reported to increase ovulation spaced approximately two days apart. After this the females may be mated if fertilised embryos are required, but not if we need eggs for IVF. The female will then be killed and the eggs or embryos harvested.

Sterile male mice will be produced either by surgical vasectomy or by breeding a genetically altered colony known as Prm1 which is maintained in the facility. Half of the animals bred carry a dominant allele for overexpression of the Prm1 fusion protein, the males that carry the modification are sterile. The alteration is inherited from the females which are fertile. Following discussions with AWERB, it has been decided to phase out the Prm1 colony and replace with surgically vasectomised males as from a 3Rs perspective, it is felt that the reduction in numbers outweighs the refinement of no surgery.

All surgery will be carried out under general anaesthetic, mice undergoing surgery will get pain relief at the time of surgery and afterwards. Embryo recipients will have embryos implanted into their reproductive tract surgically or non-surgically. Surgical vasectomies will be performed using the most refined current method which is via the scrotum.

What are the expected impacts and/or adverse effects for the animals during your project?

Most of the animals will not experience more than mild severity. Although surgery is a moderate procedure, the animals are expected to recover promptly.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Two of the four protocols, 2 and 3 are classed as moderate, because they involve surgery, embryo transfers and vasectomies which may cause short lived post-operative pain or discomfort. This is expected to be less than 5% of the total number of mice used on this licence.

The remaining protocols are classed as mild, super-ovulation usually involves two injections of hormones which may cause mild transient discomfort. Superovulation will apply to approximately 8% of the mice used on this licence. The remaining 87% will be on a mild genetically modified breeding protocol and the majority are expected to experience no harm from their genetic alteration. A small number may require ear biopsies for genotyping.

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?

This is a service licence concerned with the breeding and creation of GA lines for research, which can only be achieved by using live mice.

Which non-animal alternatives did you consider for use in this project?

Wherever possible non-regulated organisms, cell, tissue and embryo culture are used to learn as much as possible about molecular and cellular mechanisms before experiments using mice are performed.

Why were they not suitable?

The Institute's research relies in part on customised mouse models and the aims of this project are to create, breed and supply them.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 used for breeding and maintenance of the immunodeficient mice has come from data extracted from the Mouse Colony Management system of the breeding performance of the colonies we maintain. I used data collected over several years on how long a stud male can be kept before his mating performance declines to estimate the number of vasectomies required or the number of sterile mice we would need to breed.

To work out how many super-ovulations we would need to do, we looked at how many quality control tests we have done over the previous licence and approximately how many we would need to do in the future based on how demand has been changing over time.

The number of embryo transfers is based on what proportion of the frozen stocks needed to be tested in-vivo in the past plus an expectation that work for external clients may

increase in the future.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

When quality control testing frozen sperm by IVF we always have more samples on standby than we expect to use. If we have an unusually good superovulation, we can thaw the extra samples and test them as well as the planned ones. This means that we can get more tests completed with the same number of mice, so using fewer mice per test.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Super-ovulations are planned so that we use females of optimum age and weight to get the maximum number of high-quality oocytes from the smallest numbers of animals.

When selecting studs, males are picked between 2 and 6 months of age for optimum sperm production. To avoid wasting super-ovulated females by mating them to potentially sterile males, all studs are tested for fertility beforehand.

Numbers of embryos implanted per recipient female are carefully calculated to ensure the fewest number of transfers results in the highest number of healthy live offspring.

Following discussion with AWERB, it has been decided that although using the Prm1 colony is a refinement compared to vasectomies, the extra breeding of genetically altered animals and associated wild types outweighs the benefits of fewer moderate vasectomy surgeries. The Prm1 colony will be phased out which means that there will be a considerable reduction in the number of animals used on the breeding and maintenance protocol compared to the previous licence. We will therefore cryopreserve embryos from the colony, to supply other establishments if requested, or to revive the colony if the reduction/refinement cost benefit analysis changes in the future.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Surgery is performed by a small team of skilled technicians with excellent success rates. Analgesia is always given to animals undergoing surgery. Numbers of embryos implanted are optimised for the maximum numbers of healthy pups born from the fewest procedures.

Procedure success is monitored and reviewed, technicians receive regular refresher training to minimise the distress to the animals and ensure consistent results.

We have to maintain a group of sterile males because female mice will not maintain a

pregnancy with implanted embryos unless they have been mated. The sterile males can be produced surgically or by breeding a strain of mice where 50% of the males are sterile. We can tell which males are sterile by coat colour, so we don't need to take tissue samples to genotype them. This colony does not show any harmful effects from the alteration.

When surgical vasectomies are performed we use the least invasive technique possible.

Some of our mice are immunodeficient, they are kept in a clean environment where the risk of them being exposed to disease causing organisms is low, so even though they do not have a fully functioning immune system this does not cause them suffering or distress. In a small number of strains, genetic modification may adversely affect animal welfare. Effects due to genetic alteration in strains authorised for this licence will be no more than mild. Close health monitoring provision of appropriate treatment under the guidance of the NVS and adapting husbandry routines to the needs of the animal will be used to ameliorate these effects.

Why can’t you use animals that are less sentient?

We breed immunodeficient mice as a service to other projects who could not do their work on immune systems without live animals.

We replace the use of animals with cultured embryos whenever possible, however we will need to breed mice with genetic modifications. Although it is possible to test embryos for the presence or absence of a particular DNA (deoxy-ribonucleic acid) sequence it is not possible to determine how this affects gene function without studying live animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Mice are picked up using non-tail handling methods which reduces stress.

All surgery will be performed aseptically. Mice undergoing surgery are kept warm during and after surgery with thermostatically controlled warming plates. Peri-operative analgesia will be given and maintained after surgery as long as is necessary to alleviate pain. Animals also receive an injection of warm saline solution to help post-surgical recovery. They also have opthalmic gel applied to protect their corneas, this can interfere with the grimace scale to measure distress. Instead our technicians are experienced in spotting changes in body posture or behaviour which would indicate distress.

Methods for producing genetically altered animals are constantly being developed. Any widely accepted techniques and innovation may be implemented when these would result in refinements of current practices. When advised of potential refinements we will seek advice from the NIO and if necessary seek additional training in order to incorporate them into our current protocols.

Immunocompromised strains will be kept in appropriate bio-secure housing such as isolators or IVCs

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

HO Minimum Standards for Aseptic Surgery. LASA Guiding Principles for Preparing and

Undertaking Aseptic Surgery.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We get regular updates from NC3Rs on advances in reduction refinement and replacement. Our NIO produces a monthly newsletter and advises on how these can be applied in our work.

66. Coccidia control methods in poultry

Project duration

5 years 0 months

Project purpose

- Basic Research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Coccidia, Parasites, Control

Animal types	Life stages
Domestic fowl (Gallus gallus domesticus)	neonate, juvenile, adult
Turkey	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 provide supportive data for regulatory purposes on the safety, efficacy and quality of anti-coccidial vaccines, treatment products, and disinfectants in poultry for customers, pharmaceutical and disinfectant companies.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Coccidiosis is one of the most important poultry diseases on a global scale. Coccidiosis thrives in high population/ density farmed poultry and causes sub-clinical impacts such as decreasing weight gain, decrease of performance, and an increase in feed conversion ratio and clinical signs such as mortality. The cost of Coccidiosis to the UK poultry industry has been estimated to be between £73.0-£125.5 million in 2016 alone (Re-calculating the cost of coccidiosis in chickens – Blake et al 2020).This licence will have a direct impact on the welfare of commercial poultry as well as economic impacts for producers.

What outputs do you think you will see at the end of this project?

Evaluation of efficacy of new anti-coccidial treatments.

Evaluation of vaccine efficacy, including continuation, and developmental stages.

Identification of resistance/sensitivity of current non-efficacious anti-coccidial products against field strains.

Evaluation of new efficacious disinfectant candidates against coccidia oocysts.

Maintainance of sensitive reference strains of Eimeria.

Who or what will benefit from these outputs, and how?

By offering a service which can provide safety, efficacy and quality data on anti-coccidial vaccines, treatments and disinfectants, effective products can be identified and brought to market resulting in an economic and welfare benefit to the poultry industry and animals respectively. With the exception of identifying resistance in field strains of current products, it is expected that impacts derived from each project within this program of work will not be fully realised until the completion of the project due to the length of the product lifecycle of candidates tested.

How will you look to maximise the outputs of this work?

Outputs will be maximised through providing this as a service to industry partners including pharmaceutical and disinfectant companies. By working with industrial partners, we aim to maximise the likelihood of developing new effective anti-coccidials and disinfectants to improve welfare of farmed poultry.

Where applicable, any projects demonstrating refinement in the prevention or treatment of coccidia will be published in relevant scientific journals or presented at relevant national and international conferences to the wider scientific and poultry farming community.

Species and numbers of animals expected to be used

- Domestic fowl (Gallus gallus domesticus): 5000
- 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.

Eimeria are obligate and can be host-specific. In order to maintain a library of viable strains, appropriate poultry species are required. The use of each species within a project however, will be dictated by the requirements of the customer and the strain of Eimeria used.

The use of 1-60 day old birds is a necessity as Coccidiosis is commonly developed within the gut wall of young birds, before natural immunity is achieved in later life stages.

Typically, what will be done to an animal used in your project?

Projects that will be carried out within this service licence will be determined by the relevant guidelines such as the European Pharmacopoeia monograph (1) and the European Food Safety Authority (EFSA) Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) (2). They will also be in compliance with regulations such as (EU) No 528/2012 of the European Parliament. Within these references, guidelines on design, size and replication of groups, including controls and the Quality Standards required are stated and will be followed during experimental design.

In most cases, poultry will be housed in grid bottomed cages to allow for faecal collection. Dimensions will be appropriate for the age, size, species and number of individuals. Poultry will be randomised into experimental groups, and each group and individual will be identifiable. In all cases, treatment groups will then have doses of sporulated coccidial oocysts administered by oral gavage or via the cloaca. Challenged poultry will typically only experience one procedure however, in order to evaluate anti- coccidial vaccines and treatments, repeat oral gavage may be required. In these cases, a minimum interval between repeat will be 1 day.

What are the expected impacts and/or adverse effects for the animals during your project?

The doses of Eimeria oocysts are calculated to induce retardation in weight gain of 15% to 20% over the period of the study compared to uninfected controls. Weight retardation is greatest over the period of active coccidiosis (approximately days 4 to 7), but there is always a recovery after this initial loss.

However, field strains can vary in pathogenicity, and great care is always taken to monitor birds infected with field isolates to ensure that the severity limit is not exceeded.

We would also expect inappetence, listlessness, and ruffled feathers approximately 4 days after dosing. These symptoms are transient, with individuals commonly recovering within 48 - 72 hours after the onset of symptoms. Any individuals exhibiting any of the above signs for longer than 72 hours will be euthanised. It is also possible that individual birds may develop more copious diarrhoea with or without blood and mucus, and these birds will be euthanised immediately.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Pre-study: It is expected that up to 5% of all chicks (Domestic Fowl, Turkey) purchased will suffer early chick mortality.
Inoculation via oral gavage: Domestic Fowl: 95% mild, 5% moderate; Turkey: 95% mild, 5% moderate.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Eimeria are host specific and require natural host animals in order to produce viable oocysts and to meet the criteria set out by the relevant European Pharmacopoeia monographs and licencing authorities.

Which non-animal alternatives did you consider for use in this project?

In vitro cell culture (3, 4).

Why were they not suitable?

Most Eimeria are host- and tissue-specific in their development. Primary cultures of avian chick kidney cells have been used to propagate the E. tenella life cycle, but the efficiency of this is far too low for purposes such as vaccine production. Replication in non-avian cell lines fails to support completion of the full life cycle. As more cell lines become available, more work is being carried out on in vitro culture. The use of chicken cell line CLEC-213 has shown the completion of the E. tenella life cycle to be possible, however, further research is needed to confirm that this is a viable alternative.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 represent the maximum number which may be used over the period of this licence. The minimum number of individuals needed for passaging Eimeria in order to obtain the required number of parasites used in the studies will be based on previous work carried out to determine viable oocyst quantities produced (Annex 1).

Furthermore, the EP monograph (3.) and other relevant guidelines, specify the numbers of birds required, and will dictate much of the other study requirements carried out under this licence. In cases where further guidance is required, statisticians will be consulted prior to the commencement of a challenge.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

To ensure the reduced number of challenged poultry, Eimeria will only be passaged to maintain stocks of fully sensitive and reference strains. Most Eimeria remain viable in a refrigerator for up to 3 months depending on species, or are able to be stored in liquid nitrogen. Consequently, passaging is only carried out when necessary. The numbers of individuals used for passaging parasites is always kept to the minimum needed to obtain the required number of viable oocysts for subsequent challenges.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

In the case of evaluating disinfectants against Eimeria oocysts, In vitro testing will be used on fresh oocysts to identify suitable candidates, only those with 95% kill of oocysts or over will progress to the in-vivo phase of testing.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

A number of Eimeria strains are obligate and host-specific. Consequently, strains require specific species of bird in order for replication to occur in viable quantities for use within a challenge. The oral gavage route for inoculation is a tried and tested method used during coccidia challenges and allows for the direct application of oocyst dosing within the challenged bird.

Why can’t you use animals that are less sentient?

Eimeria are host specific and require natural host animals from a juvenile stage before immunity can be developed, in order to allow the onset of coccidiosis and replication of viable quantities of oocyst.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Having carried out Coccidia work over 80 years, there is significant experience and knowledge of coccidia and of the interactions and effects of Eimeria at this establishment. Consequently, prior to a challenge, relevant documentation will be consulted in order to

determine accurate challenge levels so we are able to; use the minimal number of animals, cause the least harm/severity to the animals whilst still achieving a sample effect and achieve statistically accurate results. Post inoculation, birds will be observed twice daily by a competent person. The clinical condition of the birds will also be recorded on score sheets (Annex 2).

The doses of coccidial oocysts are calculated to induce retardation in weight gain of 15% to 20% over the period of the Study compared to uninfected controls. Birds are weighed before dosing, and again at intervals (this varies depending on the Study protocol) until the end of the Study, usually 7 to 14 days after challenge. Weight retardation is greatest over the period of active coccidiosis (approximately days 4 to 7), but there is always a recovery after this initial loss. However, field strains can vary in pathogenicity, and great care is always taken to monitor birds infected with field isolates to ensure that the severity limit is not exceeded. We would expect to see the following adverse effects in birds approximately 4 days after dosing: Inappetance, listlessness, weight loss, ruffled feathers and mild diarrhoea Birds are expected to recover spontaneously within a maximum of 72 hours. Any birds exhibiting any of the above signs for more than 72 hours will be euthanised. Birds showing more copious diarrhoea, with or without blood and mucus or which is more persistent, will be euthanased immediately.

Annex 2:

CONDITION CODES – CHICKEN

Study Number: **Challenge:**
Project Code: **Treatment:**
Room:

Condition	Code	Comments	No improvements
No problems (All birds active and alert)	0		
Ruffled feathers	1	Monitor over a 8 hr period (minimum of 2 checks)	**Inform SD / NACWO**
Eye condition (red , watery, third eyelid pronounced)	2	Monitor over a 8 hr period (minimum of 2 checks)	**Inform SD / NACWO**
Droopy appearance (low head carriage dropped wings)	3	Monitor over a 8 hr period (minimum of 2 checks)	**Inform SD / NACWO**
Huddled away from group	4	Monitor over a 8 hr period (minimum of 3 checks)	**Inform SD / NACWO**
Not eating/drinking	5	Inform NACWO and Monitor every 2 hrs	**Contact SD / NVS If NO Improvements within 6 hrs Euthanize**
Reluctant to move	6	Inform NACWO	**Contact SD / NVS Animals are immediately euthanased**

Condition		Code	Comments
Slight Diarrhoea	only	7A	Birds look all ok
	in combination with condition codes 1-4	7B	Increase daytime observations to every 2 hours up to 8 hrs Inform SD / NACWO
	in combination with condition codes 5 - 6	7C	Animals are immediately euthanased Inform SD/NVS
Slight blood in Faeces	only	8A	Birds look all ok
	in combination with condition codes 1-4	8B	Increase daytime observations to every 2 hours up to 8 hrs Inform SD / NACWO
	in combination with condition codes 5 - 6	8C	Animals are immediately euthanased Inform SD/NVS
Diarrhoea with copious blood		9	Animals are immediately euthanased Inform SD/NVS
Ataxia		10	Animals are immediately euthanased Inform SD/NVS
DEATH		11	Inform SD/NVS

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Using the 3Rs website (www.nc3rs.org.uk) we will consult with the ARRIVE guidelines. Guidance will also be sought from other available sources which include Defra (https://www.gov.uk/government/publications/poultry-on-farm-welfare/poultry-welfare-recommendations), the The Poultry Club of Great Britain (https://www.poultryclub.org/resources/health/), and also internally using available resources such as AST544 - Care and Welfare of Domestic fowl. Further documentation developed by on-site Teams will also be consulted regularly.

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 3Rs advances through regular visits to the 3Rs website in addition to receiving associated emails and newsletters. Consultation with the NIO, NACWO and NVS will also be used to be well informed of any potential 3Rs advances applicable to this licence. I will also ensure that any advances or new strategies are communicated and discussed with my team via meetings, emails and provide details of recommended online resources.

There are also teams on-site who's primary primary purpose is to develop clear guidance and documentation relating to animal care and welfare, rolling enrichment programmes, procedural and technical methods, and to promote the principles of the 3R’s.

67. Cortical circuits in sensory behavior and learning

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Brain activity, Learning, Behavior, Sensation

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to understand how the circuitry of the cerebral cortex, a major part of the brain, enables learning and performance of sensory behaviors.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 cerebral cortex is a critical part of the brain for our behavior, from sensing our world, applying learned knowledge, making decisions, to executing movements. The cortex achieves its abilities using stereotyped circuitry that is repeated across the surface of the brain. All cortical areas—whether associated with touch, sight, hearing, taste, movement, or planning—appear to contain a similar set of cell types, similarly organized into distinct layers, with stereotypical patterns of connections among layers and with other parts of the nervous system. Our studies will identify how different cell types and different circuits of these cells contribute to our abilities to learn and perform sensory-guided behaviors.

Many mental disorders are diseases of cortical circuits. Understanding how these circuits work will facilitate our ability to design therapeutic treatments for various disorders. For

instance, different cell types and circuits are affected distinctly differently by drugs, and understanding how they work will enable rationale design of pharmacological treatments. Similarly, knowledge about these circuits can improve brain-machine interfaces to restore senses (touch, sight, hearing) or movement to patients. Additionally, our presently limited understanding of the architecture of healthy cortical circuits has inspired the design of intelligent systems with numerous practical applications. Systems that benefit society will be improved as we learn more about the human brain's solutions to complex problems.

What outputs do you think you will see at the end of this project?

The primary output of this research will be increasing knowledge through peer-reviewed journal publications. We hope to make discoveries on two main topics.

First, we aim to understand how the microcircuitry of cortex – the detailed wiring between specific cell types – enables the brain to perceive different objects in the world and make decisions about how to act accordingly. Presently, the field's knowledge largely concerns how different areas of the cortex might contribute to perception and decision making, not how the microcircuitry contributes to this.

Second, we aim to understand how these circuits change during learning. This will reveal which cells and connections are the site of learned information and which cellular mechanisms facilitate learning new behaviors.

Who or what will benefit from these outputs, and how?

Over the course of the project and for some years thereafter, our results will primarily benefit the scientific community. The project will identify fundamental principles of how the cerebral cortex enables behavior and will guide the development of further scientific research by many others. Our findings will also be of interest to the general public. Behavior and learning are easily relatable to all people, and the public interest in mental health has grown significantly.

An important byproduct of our research has always been the enrichment of doctoral and medical students and young postdoctoral researchers involved in the research. They will receive conceptual training (e.g., problem solving, project design, project management), technical skills (e.g., surgical skills, programming, data analysis), and communication skills (written and spoken) that will serve them in future careers in academia, industry, medicine, and policy.

In the longer term, we envision our work benefiting patients by creating targets for novel treatments. Because we focus on general principles of the brain and the interaction between brain regions, we hope this aspect will be relatively wide-reaching. In other words, we hope to benefit future treatment of neurological and psychiatric disorders, many of which involve dysfunction of the cortex, such as schizophrenia, attention deficit hyperactivity disorder, autism spectrum disorder, and stroke.

How will you look to maximise the outputs of this work?

Most of our publications in the last 15 years have involved collaborations that allow us to make discoveries that we alone could not achieve. We intend to continue in such collaborative endeavors.

Our work is disseminated through a variety of means, including journal publications, presentations at scientific conferences, social media, and outreach efforts. The lab also has a history of publishing preprints (on BioRxiv) since this option became available. We also publish data and code for our papers online.

The lab is widely known in neuroscience for publishing negative results, which due to human cognitive biases many people would normally consider “unsuccessful”. Many of our papers demonstrate how one can learn just as much from negative results as positive ones.

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.

The species in question, mouse (Mus musculus), was chosen for multiple reasons. This research project seeks to uncover general principles by which the cerebral cortex enables behavior and learning. The genomes of mice and humans are strongly similar, and virtually all of the architectural features of the mouse cortex are recapitulated in humans. We know far less about the connectivity and dynamics of cortical circuits in virtually all other species. Mice are also a very practical choice for these experiments due to their small size and amenability to invasive imaging/recording of large areas of the brain. Numerous genetically altered mouse lines for sensing activity in and manipulating cortical circuitry are available, making them a particularly compelling option.

Mice explore their environments with their whiskers, discriminating textures and shapes similarly to humans using their fingertips. We have designed an extensive set of tasks that use entirely natural, non-noxious stimuli (textures and shapes) to study how cortical circuitry in the mouse whisker system enables learning and behavior.

Our studies are concerned with normal behavior in adulthood. We therefore use adults, rather than neonates, juveniles or aged mice.

Typically, what will be done to an animal used in your project?

Mice will undergo a surgery under general anesthesia in which we will implant a small head plate that allows us to hold them in a fixed position during behavioral training and testing, brain imaging and recording, and/or neuronal manipulations. Depending on the scientific question, we may additionally perform one or more of the following:

- implant a transparent glass window in the skull to allow imaging of cortex

- implant a special lens that allows imaging of deep structures beneath cortex (e.g., thalamus)

- make a small opening in the skull that allows administration of various substances or insertion of fine microelectrodes

These additional procedures may be done in the same surgery as head plate implantation or in separate surgeries with full recovery between each procedure.

After at least 5 days of recovery, mice will be habituated to consuming their daily water at a controlled time rather than from a water bottle in their home cage. They will also be habituated to human handling as well as head fixation in experimental setups.

Mice will then be trained daily in head-fixed behavioral tasks that use entirely non-noxious stimuli. This typically involves an automated system presenting a small object close enough to the mouse so that they can actively touch it with their large facial whiskers. Mice may respond to or classify objects by licking one or two ports to receive a small water reward. Behavioral sessions typically last less than 1 hour, during which mice perform hundreds of behavioral trials. Our simplest behavioral tasks are learned in 1-2 weeks whereas our most complex behaviors may require up to 2-3 months of training. After the mice reach a high level of performance on the task, we record/image and/or manipulate brain activity as trained mice perform the task. In some experiments, recordings/manipulation may require a brief surgery while still under water restriction to allow access to the brain (e.g., by recording electrodes) and recordings/manipulations the following day during behavior. In studies of learning, brain activity is monitored or manipulated as mice transition from novice to expert stages of performance.

The daily water amount for each mouse will be calculated based on weight, which will either be attained in full during task performance or topped up after training. The weight of all mice will be monitored daily while they are on water restriction, with water amounts being adjusted as necessary to keep the weight within a target percentage of their starting weight.

After the experiments are completed (typically over a period of 1 week following behavioral training), the mouse is killed, and the brain recovered for anatomical analyses.

What are the expected impacts and/or adverse effects for the animals during your project?

Transient post-operative pain after a surgery is expected and therefore controlled for with pain relieving medications.

Water restriction for our behavioral paradigms typically cause mice to lose 10-20% of their body weight. Some weight loss normally lasts throughout behavioral training and testing, which ranges from 1 week to 3 months depending on the experiment. Typically animals recover some weight in the course of the experiment.

In some experiments, we perform a brief (typically 30-minute) surgery on water restricted mice to make a small craniotomy for recording/manipulating neurons. (The cranial surgery is performed at this time because we need mice to achieve full performance on the behavior and continue to perform the behavior during recording/manipulation, and mice would require extra days of retraining if removed from water restriction. Leaving the craniotomy open, even if plugged, for a long recovery then retraining period would be dangerous, inviting infection.) In our experience, water-restricted mice tolerate these brief surgeries well, with little or no noticeable difference in the rate of adverse effects

compared to mice off water restriction. The day after a brief surgery, restricted mice also perform our behavioral tasks at pre-surgical levels.

Expected severity categories and the 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% Subthreshold, 40% 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?

The phenomena under study--cortical mechanisms of sensation, perception, and learning--are dynamic processes that occur only in intact brains. As we are specifically addressing how small microcircuits in cortex mediate these phenomena, we need to acquire data from large groups of individual neurons. The necessary intracranial surgeries and neural manipulations in a conscious animal cannot be performed in healthy human subjects. Methods to non-invasively study humans are not able to monitor individual neurons and small neuronal circuits.

Which non-animal alternatives did you consider for use in this project?

We have considered in vitro preparations, computer simulations, and non-invasive human studies.

Why were they not suitable?

At present, too little is known about the relevant anatomy, physiology, and cellular properties of brain circuitry to study these phenomena by computational modeling. In vitro studies (isolated organs, organoids, cultures, etc) cannot be used to address behavior or neural circuitry underlying particular behaviors, precisely because they lack behavior.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of mice is based on sample size calculations we performed for typical experiments in preparing the applications for the award funding this work. These numbers are highly consistent with our published past studies. It is also consistent with values from other laboratories performing similar experiments around the world, based on literature searches and conferences involving other laboratories. This number represents the estimated number of experimental mice necessary to generate statistically significant results, factoring in the expected fractions of mouse pups that will have desired transgenes.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have identified, used, and published multiple genetically engineered mouse lines that are highly appropriate for these studies. Through the literature and scientific conferences, we regularly monitor for new lines that could further improve experimental design and data collection.

We have consulted the NC3R's Experimental Design Assistant as a tool for planning our experiments.

Large-scale recording techniques, such as array recording and two-photon microscopy can provide tens to hundreds of neurons from a single mouse. This high throughput has been extremely effective in reducing animal numbers. Additionally, we often leverage internally paired comparisons to enhance statistical power and further reduce numbers.

Individual mice may also afford the opportunity to gain multiple kinds of data towards different questions. For example, we recently published a study in which we obtained behavioral data, electrophysiology data, and optogenetic manipulation all from the same mice. Each dataset was interesting and analyzable in its own right.

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.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 make use of both wild type and genetically engineered mice that are group

housed to provide social interaction and stimulation. Genetically altered mouse lines enable the targeting of proteins to report or manipulate activity to specific cell types. The genetic alterations we use are harmless to the animals and provide the same quality of life as experienced by wild type mice. These genetically altered animals also reduce or eliminate the need for additional surgical procedures, such as injections of viruses to deliver genetic modifications. Any surgical procedures always involve deep anesthesia as well as analgesics during post-operative recovery.

Water restriction is used to motivate mice to perform tasks having been proven both minimally distressful to the mice and effective for behavioural training. Mice are given appetive, positive reinforcers (water rewards) for correct answers on a task. Mice will perform hundreds of trials in behavioral sessions for such rewards. Our behavioral tasks do not involve any aversive, negative reinforcers (e.g., electric shocks, air puffs to the eye, noxious tastants/odorants, etc). Water-restricted mice do lose weight initially, but typically tolerate water restriction very well. We and others have shown that mice perform sophisticated behavioral trials even at 80-85% of their pre-restriction weight. Most mice recover much of their pre-restriction weight as they become experts on a task. Body weight provides a robust measure of health before any more serious signs of dehydration are observed (e.g. hunched posture or piloerection). This allows us to provide supplementary water as necessary to prevent adverse effects.
Recently, promising alternatives to water restriction have been used in other behavioral tasks (e.g., visual tasks, freely moving behaviors). We will pilot combining one such alternative with our head-fixed tactile discrimination tasks.

Beyond initial implantation surgeries, the methods are relatively non-invasive. Cellular imaging and optogenetics only require shining light on the head. Modern microelectrodes, when necessary, are so small (only 1 or 2 cell bodies thick) that they do not leave detectable damage in the brain.

Why can’t you use animals that are less sentient?

The mouse is one of the lowest-order mammalian species that engage in complex sensory behavior and learning. The major organizational features of the mouse brain are also found in the human brain. Animals less sentient than mice, such as invertebrates, lack cortical layers and cannot be used to investigate the architecture of the mammalian cerebral cortex. Invertebrates do not exhibit complex sensory behavior and learning to the degree of mammals, the topics of investigation here. Similarly, anesthetized mice do not exhibit complex sensory behavior and learning.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We closely monitor the health of our mice on a daily basis. For mice previously having undergone surgery, we in consultation with the NVS provide additional measures for any mice with specific needs, including moist food to ensure weight maintenance, and medication to relieve pain or clean and heal wounds. For mice under water restriction, we weigh them daily and look for signs of dehydration. We will increase their water or remove from water restriction as necessary. Mice are habituated to human handling and head fixation for progressively longer periods before experiments begin to minimize stress.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the LASA, ARRIVE, and PREPARE guidelines to ensure best practices for refinement. Our published studies used virtually all of the recommendations of the UK's N3CRs recent working group report on head fixation and fluid control (Barkus C et al 2022 J.Neuro.Methods), even though our papers predated this report. We will continue to mirror these guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

There are continuous improvements and innovations in experimental protocols and technology to reduce and refine animal numbers. We have previously operated at the cutting-edge of these methods, and we will continue to adopt any new approaches that allows us to improve in the 3Rs.

We will also engage with ongoing institutional and national 3R’s efforts, including establishment welfare meetings and 3R’s days, interacting with the NC3R’s regional manager and the Named Information Officer, and signing up to the NC3R’s newsletter.

68. Development of tissue engineered blood vessel as vascular bypass graft

Project duration

3 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
- 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

Tissue engineering, Vascular grafting, Bypass surgery, Vascular disease model, Vascular function

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project licence is to develop a human cell-based tissue engineered blood vessel (TEBV) and evaluate the in vivo adaptation/performance in a mouse vascular graft model. The proposed TEBV will provide a novel solution to the clinical shortage of autologous vascular bypass graft and an innovative human vascular disease model for biomedical research and new drug development.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could

be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Clinical implication. Vascular reconstructive surgery has multiple clinical applications to treat life- threatening cardiovascular disease (e.g. coronary heart disease and critical limb ischaemia) and create vascular access for haemodialysis (for end-stage kidney disease). Ideally, healthy blood vessel will be taken from another part of the patient to be used as an autologous graft. However, many patients do not have suitable blood vessels due to extensive cardiovascular disease burden, or have insufficient number of blood vessels for multiple/repeated surgeries. The shortage of autologous vascular grafts leads to application of synthetic vascular grafts (e.g. ePTFE and Dacron grafts) which work well for reconstruction of big arteries, such as the aorta, but are associated with high incidence of graft failure when used as substitutes for smaller vascular grafts (diameter <6mm) including coronary bypass graft, lower limb bypass graft and arteriovenous graft for haemodialysis. This unmet clinical need stimulates research into vascular tissue engineering of small calibre vascular grafts. However, previous designs of TEBV have failed to replicate the mechanical and functional features of natural blood vessels, and have been associated with a high incidence of thrombosis, stenosis and aneurysm.

This project develops cross-disciplinary approaches to fabricate a human cell-based TEBV that replicates the morphology, mechanics and function of the natural artery which is known to be the best vascular candidate for bypass surgery. Evaluation and characterisation of the in vivo performance of TEBV in a well-established rodent vascular graft model is a critical step of early-stage preclinical development to pave the way towards large animal trial and clinical translation. Successful development of such a TEBV promises a potential new treatment for life-threating vascular diseases and will particularly benefit elder patients with poor healing capability and young children who require vascular implants that will “grow” with the body.

Research implication. Biomedical research and new drug development are still heavily dependent on animal models. However, the species barrier is the major hurdle of clinical translation. The proposed TEBV offers an innovative human blood vessel model that bypasses the species barrier, therefore holding higher translational value and significant 3Rs impact. In addition, the tissue engineering method allows using various cell sources (e.g. aged, diabetic, genetically modified, and stem cell- derived vascular cells) to develop human vascular disease models that are not currently available.

What outputs do you think you will see at the end of this project?

By the end of this project, we expect to have a well-validated human cell based TEBV that is ready for scaling up for large animal trials. Relevant work will be published in academic journals and presented on international conferences. New IPs, patents and new collaborations with pharmaceutical industry may be developed during the research project.

The TEBV fabrication method and human/rodent xenografting animal model established in this project will be used to support further development of innovative human vascular disease models by using commercial vascular cells from diabetic and atherosclerotic patients. This is expected to both reduce but also – eventually - replace many current animal models of vascular disease and lead to new academic collaborations, research activity, and new drug discovery.

Who or what will benefit from these outputs, and how?

Short term: Animal models (including transgenic, surgical and pharmacological intervention models) are main stream research tools to study human disease. The proposed TEBV will provide an innovative research platform enabling fast construction of human arteries that can be used as in vitro models (perfusion culture) to replace in vitro animal models, and xenograft in vivo models to reduce usage of animals.

Medium term: Cell-specific genetic modifications (GM) in cultured cell lines are easy and cheap. Development of TEBVs based on these cell lines is expected to be widely used as models for mechanistic studies, therefore reducing the need for development/breeding of genetically modified animal models.

Long term: Once proven reliable, the TEBV models should be a powerful platform for new drug screening, toxicology studies and medical device testing (e.g., new stents) that bypasses the species barrier, therefore promising higher success rates in these research fields, particularly in new drug development/screening. This, in turn, will attract the pharmaceutical industry to adopt these models more widely, further reducing the use of experimental animals in research.

How will you look to maximise the outputs of this work?

I will publish research findings in relevant scientific journals and disseminate them at national and international conferences, workshops, and seminars. All the publications will be uploaded to the web server of my host establishment to promote open access to this research programme. I will attend and contribute to relevant academic society's events and activities. I will apply for the network funding to organise academic workshop around this research and promote cross-institutional communication and collaboration. I will also work closely with clinical and industrial partners to push for the next stage development towards clinical translation and commercialisation.

Public engagement and outreach will also be integrated into my research. This work will benefit from the support from my host establishment including access to training, guidance and connections with local partners. Through "Images of Research", new and dramatic scientific images create a powerful medium with which to demonstrate the importance and relevance of research work being undertaken. These images are exhibited in various public venues throughout the year. Exploration’s schools programme brings researchers into local schools with hands on activities based on cutting-edge research. My host establishment has a long-term commitment to supporting public engagement, having signed up to the Concordat for Engaging the Public with Research in 2010 and the Concordat on Openness, and has been awarded a Silver Engage Watermark in recognition of this support. This commitment takes engagement beyond academics and industry, creating dialogue with the general public and account for our research and for the use of future technologies.

Species and numbers of animals expected to be used

- Mice: 130

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures,

including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We propose to study TEBV in a well-established mouse vascular graft model that has been widely used to investigate vascular graft remodelling/failure. Mice at 10-20 weeks of age are sexually mature and fully grown, therefore can be used to model adult humans. The body weight at this age (ideally>=30g) provides good-size blood vessels and helps to reduce the surgical difficulty.

Typically, what will be done to an animal used in your project?

The project requires the use of donor and recipient animals. Donor animals will be humanely killed before suitable vascular grafts (typically the aorta and vena cava) are harvested from them and preserved (as an allogenic graft). Recipient animals will undergo vascular graft surgery under general anaesthesia and be allowed to recover for 8 weeks after which period they will be humanely killed for tissue harvesting.

The vascular graft surgery consists of inserting a TEBV or an allogenic blood vessel into the right common carotid artery of an immune-compromised mouse (these mice are less likely to reject the vessel). To insert the vascular graft, the main artery of the recipient mouse will cut in the middle and the new vascular graft will be inserted and connected to the both open ends of the carotid artery using a well-established microsurgical technique.

What are the expected impacts and/or adverse effects for the animals during your project?

Recipient animals are expected to experience some pain/discomfort for 48-72 hours following the surgical procedure but this will be minimised by good technique, good peri-operative care and the administration of analgesics.

Post operatively we will closely monitor body weight (animals are expected to lose a small % of weight after surgery but to recover this loss within days), general appearance (e.g., posture, coat condition) and specific signs of pain/discomfort (e.g., "pain faces", "twitching") and with the help of animal care staff and NVS we will adjust and revise our peri-operative care as necessary.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The severity is expected to be Moderate for all the graft-recipient 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?

It is very important to characterise and investigate the in vivo performance of TEBV in animal models. This will provide foundational data to support next stage development using large animal models and future clinical translation.

Which non-animal alternatives did you consider for use in this project?

My group has an advanced perfusion culture system (Organ-on-chip system, PreciGemone Inc.) to conduct in vitro studies on TEBV. This will ensure the TEBV meets the biomechanical request of in vivo implantation and pre-trained for the arterial pulsative flow pattern, therefore minimise the chance of development of any acute graft failure. We have checked literature that there is no alternative method can completely replace the in vivo model.

Why were they not suitable?

The perfusion culture system provides biomimetic pulsative flow to perfuse the TEBV. This system will be used to investigate biomechanics and conduct hemodynamic training to TEBV before being grafted to the animal model, but it is not suitable to study the in vivo remodelling and function of TEBV, including thrombosis, chronic neointima formation, accelerated atherosclerosis, vascular inflammation, immune rejection, etc., all of which are critical for understanding the in vivo performance of TEBV and can only be investigated using 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 our animal numbers based on our previous work on the mouse vascular graft model. We have used the minimum number of animals we need to show meaningful statistical differences between experimental and control groups.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have made use of the ‘NC3R's Experimental Design Assistant’ and have also taken advice from a biostatistician of my host establishment.

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 employ a number of different methods and parameters to analyse each sample, therefore to maximise the readout information. We will share unused tissue from sacrificed

animals with other research groups whenever possible.

We will endeavour to maximize grafting material from every donor animal such that one donor will hopefully be able to supply us with enough tissue to graft onto 2 recipients. We will employ a number of different methods and parameters to analyse each sample, therefore maximising the readout information. We will share unused tissue from all animals with other research groups whenever possible.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use a well-established mouse model of vascular grafting to study the in vivo performance of human cell-based TEBV. This mouse model is the most popular vascular grafting models for this type of research. Compared to larger animal models (e.g. dog or rabbit), the graft recipient mouse recovers much faster following surgery and tends to have fewer post-operative complications. With a target of clinical translation, we aim to directly investigate the human vascular cell behaviour in the bioengineered graft. Using immune compromised mouse as graft recipient helps to avoid acute xenograft rejection. By contrast, using rodent cell-based TEBV is not optimal for our research aim. In addition, rodent cell-based TEBV will still be allograft, rather than autograft, therefore can not completely avoid host rejection. Although the actual surgical technique is well established, the use of severely immunocompromised mice and the novel TEBV are not. Hence, we will use a staged approach, using small pilot groups of only a few animals and only proceed with larger numbers if and when it is clear that there are no acute complications.

Why can’t you use animals that are less sentient?

This study aims to study the in vivo performance of TEBVs in a well-characterized mammalian species that genetically, anatomically and physiologically resembles humans and can recapitulate many of the complex cellular/organ/systems interactions that impact vascular engraftments. There isn’t a less sentient species offering those advantages. We need to use adult mice at 12 weeks or older to model adult human patients and we also need to let them recover for a reasonable period of time (8 weeks) to ensure the success of the engraftment and evaluate its long-term stability.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Surgery will be performed with great care and by skilful staff and we will implement best peri-operative care in consultation with the NVS (e.g., heat support to prevent hypothermia, treats to aid recovery, appropriate analgesia, etc). Animals will be closely monitored, especially during the first hours/days after surgery and early humane end-points applied if necessary.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

This model was first developed and standardised in King's College London in 1998. Since then, it has been adopted by many research groups across the world including our lab. The surgical success rate is >95% in our group.

Any relevant NC3R publications within the cardiovascular field will be adhered to. We will also take regular advice from colleagues who are working with the same model at other institutions in the UK, plus from our local named persons.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

As mentioned above, we already work with bioengineered tissue in vitro and we expect that one of the research outcomes of this human-cell-based TEBV will be the development of novel human vascular disease models (e.g. in vitro tissue culture model or xenograft animal model) that will replace or reduce animal usage.

We regularly visit the NC3Rs website and we closely liaise with our NVS, NIO, NACWO and NTCO; we also keep abreast of the literature in this field of research, including the 3Rs.

69. Discovering new ways to treat fungal meningitis

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Fungus, Infection, Brain, Meningitis, Cryptococcus

Animal types	Life stages
Mice	adult, pregnant, juvenile, neonate

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Identify new strategies of activating brain-resident myeloid cells to help fight fungal meningitis and associated brain inflammation.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Cryptococcal meningitis is the most common cause of fungal brain infection in humans leading to:

~100,000 deaths every year. The World Health Organisation recently identified the pathogen responsible for this disease (Cryptococcus neoformans) as the top priority fungal pathogen requiring attention for research, health and policy. We therefore urgently require better treatments for this infection, which will rely on a basic understanding of how the immune system mounts a defence to this fungus within the target organ (brain) and how the fungus establishes brain infection.

What outputs do you think you will see at the end of this project?

The main output from this research will be publications in journals and books, which is the primary way in which we disseminate our findings. Publishing our work will help others in the medical mycology and wider immunology fields by preventing duplication of experiments, and providing new data that new projects and directions can be built on. Our main goal is to publish research that either demonstrates new ways of treating life-threatening fungal infections, and/or make discoveries about how anti-fungal immune responses are regulated that can then be used to develop new treatment strategies in the future.

Additional outputs of this work will be presentations and workshops to the public (e.g. in sciences festivals and cafes) to further disseminate our publically-funded research to a wider audience.

Who or what will benefit from these outputs, and how?

The immediate short-term benefit from this work will be for academic scientists and medical mycologists, who will be able to use the new findings we publish in the design and planning of their own research. Longer term benefits may be realised in the form of new treatment strategies and/or changes to policy in how fungal infections are managed in the clinic. The long-term goal of our work is to reduce the number of deaths and poor clinical outcome of invasive fungal infections, hence this will be considered in all our decisions and research planning during this project.

How will you look to maximise the outputs of this work?

We will publish all of our research findings including negative data. In particular, we aim to publish our novel treatment strategies regardless of outcome to prevent duplication of these efforts by other research groups. There are several journals within the medical mycology field that accept and publish these types of studies.

We are a highly collaborative research group with established international collaborators in Japan, China, Belgium, South Africa and elsewhere in the UK. Our research findings may therefore be presented in these other countries at local meetings and have so far been presented in 3 different continents by us and our collaborators. Therefore, we are experienced and open to sharing our research findings as widely as possible and utilising our established global network to do this.

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.

We need to use adult mice for these infection studies. When infected with the fungus, adult mice mimic many of the clinical features of human disease such as development of meningitis and lung granulomas. Immune deficiencies that predispose to the infection in

humans (e.g. lack of lymphocytes) also promote infection in mice that have similar immune deficiencies. Therefore, mice are an appropriate and useful model for studying this fungal infection.

Typically, what will be done to an animal used in your project?

The main infection model we will use is intravenous injection of fungal cells. This causes an acute fungal infection, primarily in the brain, and is typically terminated before 7 days post-infection. This is because development of symptoms of meningitis and severe infection are more likely after 9 days of infection.

The other infection route we will use in the intranasal model of fungal infection. Mice are first lightly anaesthetised, and fungal cells placed on the nose to be inhaled by the mouse during normal breathing. The fungal infection localises to the lungs, but may disseminate to the brain after 1 week. This model typically runs for 3-4 weeks, since development of breathing problems tends to occur after 4 weeks of infection.

Some of our current work suggests that fungal access to copper is important for determining the outcome of infection and the effectiveness of different therapies. We therefore will test how manipulating copper levels in mice affects infection outcome and immune responses. For that, mice will be fed diets containing drugs that deplete copper, or injected with the drugs instead, and then impact on copper levels first assessed before moving into infection studies.

In some experiments, mice may receive further injections to deliver sterile immune cells (in a procedure called adoptive transfer) to help us track how immune cells move around the body during infection. Mice may also receive injections of antifungal drugs and/or new treatments in studies where we aim to determine how to reduce the fungal infection within the brain by manipulating the immune system. These injections will be spaced out to allow mice to recover between procedures.

Another procedure that some of our mice will experience is a bone marrow transplant. Those mice will first be irradiated (a form of chemotherapy) which kills off cells in the bone marrow. This is then replaced with an injection of healthy bone marrow which travels through the blood back into the bone. The bone marrow cells we inject may have fluorescent markers or genetic mutations. These experiments help us track cells from the bone marrow into the fungal-infected brain, and understand how mutations in these cells impact on control of infection.

What are the expected impacts and/or adverse effects for the animals during your project?

The fungus used in our infection models primarily targets the brain and lungs. In the brain, the fungus may cause meningitis which results in swelling and abnormal behaviour in mice (such as sitting in middle of the cage and moving less). This is a humane endpoint for our model, and we have designed our experiments to generate data and end the experiment prior to the development of severe meningitis symptoms. In the lungs, the fungus may cause the formation of granulomas (growth abscess) that could make breathing more difficult. The first signs of this (such as laboured breathing) are a humane endpoint for our study and experiments are designed to limit this possibility. In most of our infection studies, mice may experience some weight loss and a drop in body condition.

Expected severity categories and the 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 our animals will experience a moderate severity, due to the cumulative effect of the infection and related procedures, such as injections to deliver antifungal drugs.

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-resident immune cells, such as microglia and macrophages, rely on signals from within the brain to maintain their functional phenotypes. This means that when these cells are removed from the brain, they quickly lose their identity. In our experiments, these brain-specific features of microglia and brain- macrophages are the main aspect we wish to study. This is because it is these features that determine how these cells respond to brain fungal infection, and how they can be activated to fight the infection using experimental treatments. For these reasons, it is essential we use mice to analyse these cells as we cannot model their brain-specific identities using in vitro systems or cell lines.

Which non-animal alternatives did you consider for use in this project?

In our lab we have considered and occasionally use immortalised microglia (cell line), but there are no cell lines available for brain-resident macrophages. Our lab has generated several RNAseq datasets that can be used to generate and test hypotheses about potential new drug targets for cryptococcal meningitis without using new mice. I am also working closely with collaborators who have developed non-animal alternatives (e.g. organotypic slice models) that may be relevant for aspects of my research. I intend to work closely with them to develop these models in a way that is useful for our work to further reduce our animal usage.

Why were they not suitable?

While microglia cell lines are available, these lack many of the features seen with primary microglia. For example, microglia cultured in vitro do not produce some cytokines and chemokines (e.g. CXCL1) whereas primary microglia are able to produce these molecules, likely because they are in contact with other types of cells within 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?

Our lab has many years experience and has published extensive data using the infection models proposed in this application. From that, we are able to estimate the variability in the parameters we intend to measure, which gives us a good idea of the number of mice needed to generate statistically robust results (around 3-6 mice per group). Based on this and the fact that we will replicate all experiments at least once for reproducibility, we have estimated enough mice to cover all of these types of experiments at the larger group size.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will collect multiple read-outs from each mouse used in our studies, and then use the data to make connections between the different read-outs. For example, we will analyse microglia immune responses and compare fungal growth in different brain regions and how these different parameters correlate (a central aim for this research). In some experiments, we will also bank isolated tissues (either as stored genetic material or frozen tissue blocks) that we can analyse at a later date once our research becomes more advanced, without having to use new mice.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Genetically-altered lines are bred so as to provide wild-type littermate controls alongside the test mice, which will ensure we do not breed more lines or mice than required. These lines are also used to generate animals for other projects so we are not duplicating breeding efforts for this project.

Pilot studies are the primary way in which we will determine mouse group sizes in future experiments. We will adjust the number of mice used per experiment (if required) should the pilot study data determine that this is needed.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The infection models used in this project have been refined by many research groups in the field over the last few decades, and we have further refined the dosage and timing in

our own group over the last 5 years. We have previously performed pilot studies to determine the optimal dose of fungal cells for the different infection routes (e.g. intravenous, intranasal) that generate a measurable infection and associated immune response without inducing significant clinical symptoms. We will continue to use these refinements in this project. We will also keep up to date with new advances in refinements to the infection models as they are published, and implement where appropriate.

Why can’t you use animals that are less sentient?

Macrophages come in many different types as they become specialised to different organs during embryonic development. Macrophages in fish or insects do not have the same level of specialisation as mammals, and this macrophage specialisation is important for how diseases develop. For that reason, we must use adult mice so that we can study how brain specialised macrophages and microglia respond to fungal infection and their role in inflammatory disease caused by this infection.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Our mice are monitored at least once daily after infection. Our group members and animal facility staff are experienced in the clinical signs of infection for these models, such as watching for changes in breathing patterns and monitoring any changes to head size/shape that may indicate meningitis. As outlined above, we have refined our models to minimise these potential outcomes by altering the dosage and timing of our models. In some of our proposed studies, mice may receive treatment with antifungal drugs and/or new treatments that would further limit the development of clinical infection.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All of our procedures (e.g. injections) will adhere to the best practice guidelines published by LASA. We will also refer to ARRIVE 2.0, use the NC3Rs experimental design tool and align with PREPARE guidelines in the design of our experiments.

The infection models we will use have been recently published and are included in best practice step- by-step protocols that are also published by our group and others.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I regularly receive 3Rs updates from local mailing lists and email alerts which disseminate information about new models, protocols and group meetings. I also set up 'google alerts' for new research of interest that is then directly emailed to me, which helps me keep up to date with new research and model refinements as they are first announced.

70. Function of basal ganglia circuits

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Neuroscience, Basal ganglia, Dopamine, Parkinson's

Animal types	Life stages
Mice	juvenile, adult, neonate, pregnant, embryo
Rats	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?

The aim of this project is to advance understanding of the role of nerve cells in a region of the brain called the basal ganglia. Dysfunction of the basal ganglia plays a role in many neurological diseases and adverse behaviours, including Parkinson’s disease and addiction. We will apply the knowledge gained in normal animals and models of Parkinson’s to help understand what goes wrong during 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?

New types of nerve cells and connections are still being identified within the brain. In order to make progress in combatting neurological disease, it is critical to understand the fundamental nature of how different groups of nerve cells in the healthy brain communicate with other brain regions and guide our behaviour.

What outputs do you think you will see at the end of this project?

It is to be expected that this work will advance knowledge by providing fundamental insight into the function of different brain regions. For many brain regions, we still do not understand: 1. The types of brain cell present and how these cells are connected into discrete circuits; 2. The properties of different brain cells; 3. How different brain cells guide behaviour. The work will generate important data to address these questions which will be published and shared (e.g. in scientific journals and online databases). The data will inform computational models and potentially the design and selection of therapies for basal ganglia disorders.

Who or what will benefit from these outputs, and how?

The primary beneficiaries will be the academic community; results from this work will advance and support other research, by providing key new information about different types of cells, how they are connected, and their functions. In addition, the work will benefit the career advancement of the research team. In the medium to long term, it is hoped that the resulting advances in knowledge will guide the rational design of new therapies (e.g. for Parkinson's).

How will you look to maximise the outputs of this work?

We will maximise outputs through collaborations with colleagues, dissemination at conferences, discussion with industrial partners, and publication of our research including sharing raw data.

Species and numbers of animals expected to be used

- Mice: 2000
- 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.

We are studying how the brain is put together and how individual brain cells are connected together in the brain to guide our thoughts and actions. It is therefore necessary to use living, conscious animals which can express relevant behaviours. We use mice and rats since they are the lowest species where the principles of organisation and operation of their basal ganglia are similar to humans. Adult animals will be used to ensure that developmental changes do not unduly influence the findings and to best match the point in the human life cycle relevant to degenerative brain conditions.

Typically, what will be done to an animal used in your project?

To enable us to record brain activity, most animals (~60%) will undergo a short surgery (~1hr) to allow us to make windows in the skull to gain access to the brain, implant tiny screws, holders and probes, then seal everything with dental cement. Animals are expected to recover quickly and will be given painkillers, palatable food, and post-

operative care. To investigate how brain activity is linked to behaviour, we use tests that make use of animals' natural curiosity to help them learn and perform the tests. In most cases we will also use motivators (e.g. make them hungry or thirsty) and give rewards during the test. To minimise any stress, we first make sure that animals are comfortable with the tester and testing environment. To record brain activity, in some animals a lead is connected to the recording device while they move around their cage or testing environment. In other cases (e.g. where special stability is needed), the head will be fixed in place and the animal allowed to freely run or rest (e.g. on a treadmill). In our experience, head-fixed animals show the same breathing and heart rates as those in the home cage and animals voluntarily come to the experimenter's hand following release from restraint.

Most experiments are carried out in normal animals, but to investigate diseases like Parkinson's we use models that mimic aspects of the disease process. This might be injecting substances that cause loss of a specific type of brain-cell, or use of a genetically altered mouse that has a disease-related change which is known to occur in humans.

At the end of the experiment, animals will be killed by a humane method .

What are the expected impacts and/or adverse effects for the animals during your project?

Animals undergoing surgery will experience some pain from the interventions which will be managed with analgesia and post-operative care and is expected to last no longer than 24 hours.

Where motivation is needed to encourage animals to learn/perform a behavioural task, levels of food/water are reduced (typically <2 weeks). Animals will experience some hunger or thirst.

In rare cases, animals may be temporarily sleep deprived (e.g. to investigate links between sleep and neurological disease). We will use a mild approach (<12 hrs/day, max 3 times per week, total max 6 times), which will result in drowsiness the following day, but no signs of stress or physical discomfort.

In our experience so far, models used to mimic aspects of neurological disorders lead to only minor adverse effects (e.g. slight disturbances in movement).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Moderate - all animals

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 the brain is put together and how individual brain cells are connected together in the brain to guide decisions and actions. It is therefore necessary to use living, conscious animals which can express relevant behaviours.

Which non-animal alternatives did you consider for use in this project?

Humans

Computational models

Non-regulated model organisms

Why were they not suitable?

High-resolution clinical investigations of the activity basal ganglia neurons in the healthy brain are not feasible, nor would they be easy to justify ethically. At present techniques possible in humans have much lower temporal and spatial acuity and are unable to address the objectives of this project.

Although computer models have provided great insights into neuronal network behaviour in recent years, the models currently available in the field are not yet sufficient to address our key objectives here. For example, there is no data on the function of different types of dopamine neurons and so models currently consider just a single type. However, data we generate is used to create and improve current models.

Non-regulated model organisms lack the complex circuit organisation and behaviours to address our scientific objectives. Organoids and minibrains do not have sufficient complexity to address the scientific questions of this programme of work.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers of animals have been estimated (using power calculations) based on our current (and projected future) research and the specific objectives defined in those projects. We use our own data (or published examples) to establish estimates of variability to determine minimum group sizes required to ensure results and analyses are sufficiently powered to be statistically robust. These estimates have been peer reviewed as part of funding proposals.

What steps did you take during the experimental design phase to reduce the number

of animals being used in this project?
For simple studies we can utilise the NC3Rs EDA but for more complex designs, we work with local experts in experiment design. Most studies are designed to use within-subject controls; this reduces the total numbers of animals as each animal can act as its own control and generate multiple data points with reduced variability. Experiments are designed to use both sexes (using sex as a blocking factor) to maximise use of animals bred for the study.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

For the majority of animals, behavioural testing is combined with recording/imaging, and where possible, multiple cells are recorded in the same animal.

Tissue is often used for multiple objectives (e.g. brains from behaviour studies are normally used for histological analysis).

Where possible efficient breeding is used to ensure all offspring can be used for experiments. For example, strategies that ensure 100% of offspring are of the correct genotype.

Computer models are constructed from data to test hypotheses and generate new predictions.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Surgery: Procedures are performed in accordance with LASA guidance. During surgery, animals will be anaesthetised, given pain relief and closely monitored to ensure rapid recovery.

Behavioural testing: To minimise stress during behavioural testing, we make sure that animals are comfortable in the testing environment and give them treats and rest periods.We use minimal levels of motivational hunger and thirst by using a staged approach to evaluate the levels of hunger/thirst required for specific tasks and by using automated systems we developed in collaboration with NC3Rs which enable us to precisely schedule and monitor fluid consumption.

Recording neural activity: Head implants for recording are small and designed to minimise impact on the animals normal behaviour in the home cage. Animals will typically be positioned upon a running wheel/treadmill or platform (e.g. in a tube). These configurations are adjustable to allow each animal to be positioned such that it can sit comfortably with a natural posture and respond to presented stimuli and to perform spontaneous and learned

behaviours.
Investigating altered sleep: The sleep deprivation approach uses naturalistic stimulation (e.g. novel objects and gentle handling) and is designed to be mild and not stress the animals.

Why can’t you use animals that are less sentient?

We primarily use mice to study the basal ganglia in health and disease since the principles of organisation and operation of the basal ganglia are similar throughout all mammals and are therefore applicable to humans. Rats will be used where an appropriate disease model is not available in mice, or when working with collaborators where the rest of a study is conducted in rats. Adult animals will be used to ensure that developmental changes do not unduly influence the finding and to best match the point in the human life cycle relevant to degenerative brain conditions. To help understand the properties of brain cells, some recordings will be made in terminally anaesthetised animals; however, to understand how brain cells guide behaviour it is critical to be able to record from brain cells in the awake adult brain during behavioural tasks.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We are constantly seeking ways to refine the procedures we use - recent examples include:

the automated systems we developed in collaboration with NC3Rs which enable us to precisely schedule and monitor fluid consumption.

custom shaped 3d-printed head implants to minimise weight and influence on natural behaviour.

refinements to habituation and motivation procedures. I chair a local working group of researchers from multiple laboratories that are developing protocols to reduce stress during behavioural training.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

I will utilise resources from organisations such as NC3Rs (https://www.nc3rs.org.uk/3rs-resources) and LASA (https://www.lasa.co.uk/current_publications/) and follow the principles of the PREPARE guidelines to ensure we are using the latest guidance on procedures including surgical approaches, analgesia and methods to recognise and manage suffering. All procedures (e.g. animal handling, dosing etc) are performed in accordance with local policies reviewed and approved by the AWERB.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I regularly attend symposia, webinars, and workshops hosted by the NC3Rs. 3Rs advances and related newsletters are regularly disseminated to PIL/PPL holders at Bristol and discussed at our user- group meetings. I also attend and present my work at national and international conferences, enabling me to keep up with the latest developments and also disseminate outcomes and refinements from our own research programmes.

71. Haematopoietic cells and the surrounding microenvironment in ageing and leukaemia

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

Blood cell production, Haematopoietic stem cells (HSCs), Leukaemia, Leukaemic stem cells (LSCs), Niche

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?

This project aims to understand how blood cells are normally produced, and how this process is perturbed during physiological ageing and haematological cancers, as acute myeloid leukaemia. It also aims to investigate blood cells surroundings with the goal of identifying new targets and generate better therapies to treat blood cancer patients.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Acute myeloid leukaemia (AML) is a rare but lethal blood cancer and although new treatments are providing hope for improved survival, the health care community is still struggling to improve the poor prognosis. The majority of patients achieve complete remission after initial therapy, but most patients develop therapy resistance. Leukaemic cells reside in the bone marrow in a specialized microenvironment, the niche, that requires a complex network of interactions across multiple cell types to regulate their function. Therapy resistance is in part due to the niche that protects and supports survival of leukaemic cells. The purpose of this project is to understand why therapy resistance is developed and investigate the role played from the niche in this process, identifying targets that might be used for the development of new therapies. This will greatly improve survival of AML patients that will be the first beneficiaries of this work.

What outputs do you think you will see at the end of this project?

Expected benefits include understanding how blood production is perturbed during leukaemia and the discovery of new treatments for patient benefit in the longer term.

Our work has clinical applications that we will investigate through collaborations with clinicians at the Establishment and other medical cancer centres worldwide.

Who or what will benefit from these outputs, and how?

The expected benefits of this project would include:

New knowledge regarding the genes needed to maintain the function of malignant stem cells, or leukaemic stem cells, that cause blood cancer. The research findings will be published in academic journals and will be of interest to scientists in the field.

Insight into the interaction between leukaemic cells and their surroundings to explore the role that they play in supporting leukaemia for identification of new therapeutic targets. These research findings will be published in academic journals and will be of interest to scientists in the field.

Identification of specific candidate in blood cancers which would be of particular interest to the pharmaceutical industry with regard to drug development and clinicians treating patients.

Evaluation of the effect of one or more candidate therapies on normal and leukaemic cells which would again be of particular interest to the pharmaceutical industry with regard to drug development and clinicians treating patients.

How will you look to maximise the outputs of this work?

Our findings will be made available to other scientists including clinicians, 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 for all to access freely.

Species and numbers of animals expected to be used

- Mice: 1300

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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, and, as mammals, have considerable genetic and biological similarities to humans with regard to their blood forming system. Other less sentient non- mammalian species, such as fruit-fly or zebra-fish, lack a haematopoietic system that is comparable in complexity and anatomy to that of Homo sapiens and have been rejected as models, therefore. Only a mammalian blood cell generation model system has the potential to accurately mimic both the anatomy and complex cell biology, including local cell-cell interactions, of normal and perturbed blood cell generation in humans. Furthermore, there is considerable experience in the wider scientific community regarding the use of mice as a model system for human diseases of the blood and many reagents exist for the accurate genetic and cell surface protein characterisation of mouse cells.

The mouse is one of the model organisms that most closely resemble humans. The human and mouse genomes are approximately the same size, and display an equivalent number of genes, which are conserved. Further, mice have genes not represented in other animal model organisms (e.g., nematode worm, and fruit fly) such as those involved in adaptive immune responses. Mice can be genetically altered, there is extensive literature concerning the topics of our investigation, and our own studies can be enhanced by combination with many complementary models developed by others in the field. Definitively, mouse models are important for placing the findings of in vitro (test tube) studies or analysis done using patients' 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 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 validated in an appropriate environment within a living body.

For our studies we need animals with a functionally mature immune system, therefore we will only use adult mice.

Typically, what will be done to an animal used in your project?

Experimental mice will undergo the following typical experiences:

Ablation of the bone marrow through lethal irradiation before performing stem cells transplantation by either injection into a vein or the thigh-bone, followed by 4 weeks recovery. Blood samples will be taken up to 8 times over a 4-month period. Mice will be killed by a Schedule 1 method.

Substance administration (daily for a maximum of 4 weeks), sublethal irradiation and bone marrow transplant, followed by up to 4 weeks recovery. Blood samples will be taken up to 8 times over a 4-month period. Mice will be killed by a Schedule 1 method.

Induction of gene expression by substance administration leading to the development of a

blood cancer over 12 months. A single bone marrow sample will be taken. Mice will then receive daily substance (given to modulate activity of the cellular mechanism of interest) administration for a maximum of 2 weeks by oral gavage or injection into the abdomen. Blood samples will be taken, from a superficial vein, up to 12 times over a 4-week period. Mice will be killed by Schedule 1 method.

What are the expected impacts and/or adverse effects for the animals during your project?

We will always aim to use the least stressful method possible for all interventions. The expected impacts (not associated with significant side effects) include transient discomfort or pain when substances are administered, after injection and after blood or bone marrow sample collection.

Mice injected with blood cancer cells will, when the disease develops, exhibit signs of disease, with fatigue due to decreased number of red blood cells (anaemia) hunched posture, poor levels of socialising and interaction. The experiment will be terminated if more than moderate anaemia develops, following measurement of haemoglobin levels.

Also, irradiation prior to bone marrow transplantation can cause transient mild anaemia, expected to last a few days. Under these circumstances, and whenever else a mouse displays features of ill health, or at the end of each 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)?

Based on our experience, using these procedures and experimental models we anticipate about 95- 98% of mice experience moderate severity, such as no more than 20% bodyweight loss. Some mice will experience the discomfort of repeated (daily) injections of therapeutic agents or oral delivery. We always aim to utilise the least stressful route of administration wherever possible.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Mice have been chosen for the study because they represent the least sentient species from which meaningful experimental data can be generated; furthermore, as mammals, there are considerable genetic and biological similarities to humans regarding their blood cell forming system. Furthermore, there is considerable experience in the wider scientific community regarding the use of mice as a model system for human haematological malignancies and many reagents exist for the characterisation of mouse cells.

Leukaemia/cancer or normal bone marrow stem cells are defined by their capacity to reconstitute leukaemic or normal haematopoiesis (the formation of blood cellular components) respectively in a secondary transplant recipient. In vitro experimental systems, and computer modelling systems, are insufficient for this purpose, because they do not provide the required environment for the development of a novel tumour, or normal haematopoietic system, which typically has complex cellular architecture, involving interactions between many different cell types.

In addition, both mouse and human leukaemia stem cells cultured in vitro rapidly lose their malignant potential and change their characteristics and properties, showing a significant dependence on the in vivo location. There is currently no adequate methodology for modelling this process in vitro, in particular the molecular and environmental factors involved. Thus, without the use of a live, whole animal experimental system, the biology of bone marrow stem cells cannot be meaningfully studied.

Which non-animal alternatives did you consider for use in this project?

Some aspects of normal and malignant stem cell maintenance can be mimicked in vitro, and we will use these methodologies when possible. In particular, we have considered the use of in vitro cultures for the maintenance and differentiation of haematopoietic stem cells, and for maintenance of malignancy-propagating cells.

Why were they not suitable?

The colony forming assay is a technique used to examine the ability of a single cell to grow into a large colony through clonal expansion (clonogenic activity). Clonogenic activity is used as an indicator of undifferentiated cancer stem cells. While this assay can be helpful to assess the early aspects of blood cell formation, it can result in a multiple cell-type growth because of the time that cells are left in culture. Then, the only way to characterize the haematopoietic stem cells is in vivo setting, with the transplantation.

Leukaemic cells arise from a pool of leukaemic stem cells (LSCs) sitting at the apex of the hierarchy, also referred to as leukaemia initiating cells (LICs) for their capacity to engraft and give rise to leukaemia when transplanted into immunocompromised recipient mice. We have been successful in maintaining some LSCs ex vivo (ex vivo refers to experiments performed on cells in an artificial environment outside the living organism with minimal alteration of natural conditions); however, they rapidly lose their leukaemogenic ability properties, emphasising the need for a better understanding of the niche support during leukaemia. While we will continue to explore the methodologies mentioned above, they cannot currently adequately replace in vivo experiments (performed in mice).

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The overall aim will be to generate models whereby a measurable effect e.g., reduction in

leukaemia or leukaemia incidence following manipulation of a gene of interest or treatment with a drug can be determined using the minimal number of animals.

Animal numbers will be estimated based on statistical evaluation of previous experiments. The experiments planned for this license are similar in nature to experiments that I have performed in my previous laboratory.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The use of mice will be minimised in several ways:

By considering on-going statistical estimation of power requirements in each of the studies, using prior results in order to use the minimum number of animals while retaining sufficient numbers for statistical significance.

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 and tumours from different sites on one mouse for both treatment and control samples.

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 mice will be optimised in several ways:
By optimising our breeding programme to maximise use of mice in experimental protocols thereby minimising the number of mice we do not require. Breeding is performed in a different project licence, by a dedicated group in our Establishment.

By doing as much preliminary work as possible in culture models in vitro and in silico analysis prior to engaging in in vivo studies.

By minimising variability in results using inbred strains to guarantee uniformity, and predictable experiment response and by housing them under similar conditions.

By performing pilot studies using a small number of mice (3/group) when no information is available in the literature so that the number of mice utilised in experiments is reduced to minimal levels.

In all new experimental models and protocols, we will establish the baseline by procuring help and advice from husbandry and veterinary staff and researchers at the Establishment but also from our experienced collaborators outside across the UK and internationally. Furthermore, we will design small pilot experiments, carried out referring to https://www.nc3rs.org.uk/conducting-pilot-study, that will allow us, where appropriate, to select the ideal cell line so fewer animals are used, to calculate the minimum number of mice/groups given the rate of expected events and to determine gravity of these.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the

procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 housed in an enriched environment and are cared for by staff trained and expert in animal monitoring and handling. For experimental approaches the least invasive route for drug treatment is always followed where possible (e.g., drinking water versus injection where a drug is orally bioavailable and stable); mice which undergo anaesthesia and bone marrow puncture are treated with fluid and analgesia to minimise risk of distress.

In detail we will use here:

Mice that undergo ageing: there is no suitable alternative currently in the ageing process investigation with the only option represented by the use of intact organisms, as the processes involved are highly complex and depend on different factors. The mice will be aged here up to two years of age, as this represents the physiological age equivalent to the human age (65-70 years) where immune function declines, blood cell production is significantly altered, and blood cancers are more frequent. As in humans, ageing leads to physiological decline, which can cause minor discomfort, but this is necessary to investigate the effects relevant to human health. All the mice will be monitored daily throughout their lives; additionally, a specific monitoring will be performed from 12 months of age to assess age-associated phenotypes, as specified in the humane endpoints specific to aging; any sores or local inflammation/irritation will be treated.

Mice that develop blood cancer: the mouse currently represents the most accurate model for the study of human blood diseases; indeed, one of the drawbacks of experiments using human leukaemia cells is their genetic heterogeneity making the study of the effects of specific genetic lesions very difficult.
For disease phenotypes it is necessary to allow the disease to develop to a clinically relevant stage, which may cause moderate suffering in the animals. However, this is necessary to obtain information relevant to human blood cancer and will be minimised by daily monitoring of the mice. In addition, mice with the potential for developing haematological phenotypes will be examined at least twice weekly for anaemia by footpad inspection: if foot pads are pale they will be killed by a schedule 1 method.

Mice that undergo bone marrow transplantation: The use of bone marrow transplantation represents the most refined methodology for the identification and characterization of haematopoietic stem cells and LICs. It is associated with transient discomfort due to irradiation required to suppress the endogenous haematopoietic cells that in many cases is a prerequisite for the transplanted cells to engraft.

Mice that are treated with pharmacological agents: To develop pharmacological strategies to reverse ageing or to treat haematological malignancies, the use of drugs is necessary. Some anti-ageing compounds may have to be administered over long periods of time; however, this is necessary to determine their ability to counteract the ageing process. In addition, some drugs that are used to treat haematological malignancies are cytotoxic, and will cause transient suffering, but no lasting harm.
However, their use is necessary their effects and to identify new therapeutic strategies.

Mice that are engrafted with human haematopoietic cells: Xenografting is the most accepted model for studying normal human and malignant cells in a mouse setting. It does not cause any significant lasting harm, except those associated with disease progression (mentioned above) in case of engraftment of malignant cells.

In the small proportion of instances where mice have lost weight (e.g., after higher doses of X-ray irradiation) mice are offered mash / a more palatable diet in addition to their usual diet and monitored more frequently (e.g., twice daily).

Why can’t you use animals that are less sentient?

Other less sentient non-mammalian species, such as fruit-fly or zebrafish, lack a haematopoietic system that is comparable in complexity and anatomy to that of humans and this is the reason why they have been rejected as models. Only a mammalian haematopoietic model system has the potential to accurately mimic both the anatomy and complex cell biology, including microenvironmental interactions, of human normal and leukaemic haematopoiesis.

Although there is published literature using human leukemia xenografts in immunodeficient zebrafish, this has covered only short-term studies and has been widely used for drug screening. The purpose of my research is understanding the impact of ageing upon leukaemia and so these models are not useful. In addition, it is not a mammalian microenvironemnt, then there are differences in this regard and zebrafish genes display only 70-80% of similarity to human genes, while the mouse is at least 85% and up to 99% for some proteins.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The techniques used have been carefully evaluated to minimise distress to the animals. Mice used in surgical procedures will be treated with anaesthesia, analgesia, and post-operative rehydration e.g., by subcutaneous injection, followed by careful observation.

In other areas, irradiation doses will be administered at a level sufficient to induce bone marrow suppression but no other long-term impact; higher doses of irradiation are delivered in split doses. Bone marrow sampling will not be performed routinely, but only when characterization of bone marrow populations is needed, but the endpoint has not been reached, so that the mouse cannot be culled before.

In studies that result in the initiation of blood cancer, mice will be closely monitored for health status and killed by a Home Office approved method when leukaemia signs are displayed.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Surgical procedures will be carried according to the LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery 2017.

Unless otherwise specified, this project will follow the "Guidelines for the welfare and use of animals in cancer research” and the administration of substances and withdrawal of blood will be undertaken using a combination of volumes, routes and frequencies that of

themselves will result in no more than transient discomfort and no lasting harm (Morton et al., Lab Animals, 35(1): 1-41 (2001); Workman P, et al. British Journal of Cancer, 102:1555-77 (2010)).

Where we use aged mice, we will refer to Wilkinson (Laboratory Animals 2020, volume 5(34)), relating to the husbandry and care of aging mice.

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, and through discussing refinements with our animal care staff and vet. I will also read the reports relating to animal welfare that are circulated within the Establishment. I will attend and contribute to our Retrospective Review and Licensees meetings and the 3Rs Poster session, all of which take place annually at our Establishment.

Sources:

NC3Rs (National Centre for the Replacement, Refinement and Reduction of Animals in Research) FRAME (Fund for the Replacement of Animals in Medical Experiments) ALTWEB - altweb.jhsph.edu (Alternatives to Animal Testing on the Web)

72. Investigations to improve the control of classical swine fever and pestivirus infections.

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes

Key words

Disease diagnosis, Virus tracing Immune response, Vaccine, Host resistance

Animal types	Life stages
Pigs	adult, juvenile
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?

The aim of this project is to gain knowledge and develop improved methods that will help control classical swine fever disease (CSF) and infections with pestiviruses.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Classical swine fever is a serious, often lethal, disease of pigs that has important economic and social consequences for the global pig industry. The disease impacts on food security and on animal welfare. Prevention and control of outbreaks of CSF require a

combination of measures including early detection by reliable diagnosis and rapid investigations to track and trace the causative agent, the classical swine fever virus (CSFV). Vaccines exist that are effective in protecting against the disease, but their use would mask the presence of the virus with current surveillance methods. This would have a detrimental impact on trade in pigs and pig products and so CSF control in disease free regions involves culling of large numbers of animals to prevent the disease spreading. Better vaccines that allow detection of infection in vaccinated herds are therefore needed. This project aims to gain knowledge and develop tools to enhance the detection of CSF by improving methods to use the genetic sequence of the virus to help trace its movements. The project also aims to characterise infection caused by novel closely related viruses in the pestivirus family. Such pestiviruses typically infect ruminants but can also infect pigs without causing detrimental effects. It is important to understand the potential for transmission of these novel viruses between species and to help ensure that infection of pigs with these viruses is not confused with infection by CSFV. The project also aims to gain knowledge on the immune mechanisms, including defining the role of specific immune cells, that make the existing vaccine protective. This will help us with our further aim to develop better vaccines that do not mask infection. Finally, we aim to investigate if pigs can be generated that would not be impacted by the disease by testing if animals that have alterations in genes known to be important for virus replication are resistant or resilient to infection with the virus.

What outputs do you think you will see at the end of this project?

The project will provide new knowledge of the extent of genetic change that occurs in the virus during multiple rounds of replication in pigs and knowledge on the disease caused by pestiviruses closely related to CSFV. Knowledge will be also obtained on the host's immune response that makes the existing vaccines so highly effective. Knowledge on the efficacy of novel candidate vaccine formulations and on the ability of genetically altered pigs to resist infection will be produced. If efficacious, these candidate vaccines and altered pigs may be expected to progress into development as products in the future. The project will produce material that will be useful for developing better diagnostic tests. The new knowledge will lead to publication outputs.

Who or what will benefit from these outputs, and how?

In the short term, the new knowledge generated will benefit the scientific community, including diagnosticians, vaccine developers and livestock improvement companies, and policy makers working to improve the control of CSF. Knowledge on what makes a vaccine effective, or that gene alterations can confer resistance to disease, will also be beneficial to the scientific community seeking to develop methods to control other livestock diseases. In the longer term improving the methods available to control CSF disease will reduce the need to kill large numbers of infected, or potentially infected, animals to stop the spread of disease if an outbreak occurs in a disease-free region. This will benefit animal welfare and the farming industry. These improved control methods will also benefit programmes to eradicate CSF from countries where it is currently present. This will also improve animal welfare and help secure sustainable food production globally.

How will you look to maximise the outputs of this work?

The new knowledge from the work will be presented at national and international conferences and published in peer reviewed scientific journals. Publications will follow the ARRIVE guidelines and unsuccessful approaches will be published. We are, and will

continue to, collaborate with both national and international scientific colleagues to maximize dissemination of knew knowledge and sharing of material generated. Policy makers will be informed of the results which will be published in final reports of the projects funding the studies.

Species and numbers of animals expected to be used

- Pigs: 345

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Pigs are the natural host of classical swine fever and the only appropriate host to use for our objectives to characterise genetic changes occurring during replication of the virus in its host and the porcine host's response to vaccination, infection, or if genetic alterations lead to disease resistance. Infection with CSFV can result in acute, chronic or late onset forms of disease, the incidence of which are affected by the age of the host. The project will typically use a model of protection against the acute form of the disease that usually occurs in juvenile to adult animals. In addition, to study the immune response juvenile to adult animals, which have a developed immune system, as opposed to immature life stages, are required.

To investigate the disease induced by ruminant pestiviruses pigs will be used. Characterisation of these viruses in sheep will be added to the project at a later date. These viruses are typically isolated from ruminants but can infect pigs. These animals are therefore the only appropriate host to achieve our scientific aim to characterise the disease caused when these hosts are infected

Typically, what will be done to an animal used in your project?

Animals will be housed in groups on straw. Typically, pigs will be inoculated with a vaccine or vaccine candidate by injection either into a muscle, under or in the skin or via an intranasal spray. Further booster immunisations may be applied. Up to 12 blood samples will be taken prior to and after immunisation to monitor immune responses to the vaccine. Some pigs will be euthanised typically no longer than 3 months after receiving the vaccines or vaccine candidates.

Other pigs, which may or may not have been previously vaccinated, will be inoculated with virus, typically by an intranasal spray. Daily rectal temperatures and, blood samples, usually at 3- or more day intervals, but occasionally at 2-day intervals, will be taken to monitor disease progression. No more than 12 blood samples in total will be taken from an animal. Nasal and rectal swab samples will be taken at no more than 2-day intervals to monitor the potential for spread of virus. Animals will be euthanised typically no more than 3 weeks after inoculation with virus.

Some other pigs will receive cell transfers intravenously, typically 3 at daily intervals, of immune cells harvested post-mortem from other vaccinated donor animals. These animals may also be challenged by intranasal inoculation of virus. To closely monitor disease

progression up to 12 blood samples, typically at 3- or more day intervals but occasionally at 2-day intervals and rectal temperatures will be taken and animals will be euthanised typically no more than 3 weeks after virus/ immune cell inoculation.

Some pigs inoculated with virus will have genetic alterations. These animals will be generated by collaborators under a different project licence.

What are the expected impacts and/or adverse effects for the animals during your project?

Pigs that are inoculated with CSFV, and which are not protected by an experimental treatment, are expected to experience early signs of CSF disease for a duration or no more than 2 weeks. This typically may include reduced appetite, a lack of usual weight gain or slight weight loss, increased temperature and fever, lethargy and weakness and a tendency to huddle together. Some animals may experience conjunctivitis, diarrhoea or constipation and incoordination. It is expected that animals will not experience the worst adverse effects for more than a period of 2 days.

Some animals inoculated with novel vaccine formulations may experience swellings at the site of the inoculation which typically subside after 1 or 2 weeks.

Pigs inoculated with ruminant pestiviruses may experience some clinical signs. Acute Border Disease infections are often inapparent or induce only mild signs such as slight fever and leukopenia.

Occasionally some Border Disease isolates have produced high fever, leukopenia, anorexia, conjunctivitis, nasal discharge, dyspnoea and diarrhoea. It is expected that the virus strains we will test will result in only mild signs but if more severe signs are induced it is expected that animals will not experience the worst adverse effects for more than a period of 2 days.

There is a low possibility of adverse effects including stress, haemorrhage, bruising, thrombosis, infection at the site of needle entry, phlebitis, scarring and nerve damage due to blood sampling. Animals will be trained to become accustomed to restraint to minimise stress and potential for these adverse effects due to sampling.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

It is expected that the majority of pigs (80%) will experience mild severity associated with sampling and early stages of CSF. A proportion (20%) of pigs may experience moderate level severity for a short period.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The complexity of the immune system and the virus host interactions that impact replication within the host means that there are no alternatives that can replicate the vast array of factors that lead a vaccine to protect an animal from infection, to mimic the response to virulent virus, to reproduce the genetic changes within the virus that occur during replication within animals or to confirm that a genetic alteration of the host results in resistance to disease.

Which non-animal alternatives did you consider for use in this project?

In vitro and ex vivo systems will be used, for example, to assess genetic changes during cell culture propagation, the immune response of individual cell types to vaccines or infection and to investigate the impact of gene alterations on virus replication in particular cell types. We have considered using precision cut slice cultures for assessment of immune responses within the tonsil.

Why were they not suitable?

It is not possible to use such ex vivo and in vitro systems to study the complex interaction of virus and all the cells that occur within the whole animal in response to vaccination or infection. An organ culture system such as precision cut slice culture for porcine tonsil would be of benefit to reduce the use of animals for some initial studies but, unfortunately, such a system does not yet exist. In addition, such organ cultures only involve an immunological organ in isolation and do not reflect the multitude of immune interactions, such as cellular migrations or soluble cytokine signals, that occur between distant sites.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Estimated numbers have been determined using our previous experience of variation that occurs in the parameters we will measure in response to vaccination or virus infection. The experimental plans for each individual study will be reviewed by peers to allow thorough examination of the experimental plans. This will include review by expert statisticians to ensure the correct number of animals are used to achieve the experimental aims.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We plan to test multiple experimental groups interrogating different vaccine formulations, or the role of specific immune cells, at the same time to minimise the number of control animals required. Small scale pilot experiments will establish the model for new virus or

hosts and for determining parameters such as the dose and formulation method of treatments before larger scale experiments. The experimental design assistant (EDA) from NC3Rs will be used for specific study designs, and a biostatistician will be consulted on the appropriate sample size prior to any study start.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

To minimise variation standard protocols will be applied for example for preparation of test treatments and challenge virus and for performing the inoculations and laboratory analysis. To control variability and allow smaller group sizes animals will be of similar age, weight, with a good health status. Tissues from the project will be archived for our own further investigations and where possible made available to other researchers

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The project involves vaccination or transfer of immune cells to pigs and infection of pigs, some of which may be genetically altered, with classical swine fever virus. We will also characterise infection of pigs with ruminant pestiviruses. CSFV infections will use strains that we have previously characterised and for which we have an established clinical scoring system, for clinical monitoring. If infection with a new strain is required a pilot experiment in small number of animals will characterise the clinical signs and inform on clinical monitoring and scoring system before large scale experiments. Definition of scientific end points appropriate to the objective of each study are used to ensure that animals are euthanised at a point that ensures they experience the least pain, suffering, distress or lasting harm needed to meet the scientific objectives. However, to demonstrate that the experimental investigations have a role in protection from disease it is unavoidable that some animals will experience discomfort associated with the early stages of CSF disease.

Why can’t you use animals that are less sentient?

The project involves models for vaccination and/or infection of pigs with classical swine fever virus or pigs with ruminant pestiviruses. Pigs are the natural host for classical swine fever and no other animal produces the same response to vaccine or infection with virus. To study the immune response, it is necessary for animals to be immunocompetent with a developed immune system and so immature stages cannot be used. Ruminant pestiviruses usually infect ruminants, such as sheep, goats or cattle, but can also infect pigs. It is not possible to achieve the scientific aim of characterising the infection caused by novel pestiviruses in pigs using other species

How will you refine the procedures you're using to minimise the welfare costs

(harms) for the animals?

Our clinical monitoring scheme includes actions to increase monitoring of animals exhibiting clinical signs. Meetings are held before and after each study between those conducting procedures, caring for and monitoring animals, NVS, NACWO and scientific staff. These meeting will identify suggestions for refinements, e.g. reviewing the clinical scoring system to refine end-point, which will be implemented.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use the Norecopa PREPARE guidelines and information from associated web sites, such as specific guidance for the use of farm animals in research.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will regularly review information from the NC3Rs web site and newsletters as well as advances in procedures resulting in 3Rs such as from colleagues in the agency or from relevant external publications which are regularly disseminated across the agency via an internal newsletter. As national an experienced laboratory we will be in regular contact with other pestivirus researchers world-wide and new knowledge on procedures of similar studies will be taken into account. Relevant advances will be incorporated into study designs and procedures.

73. Mechanisms and Applications of Targeted Protein Degradation

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Protein Degradation, Chemical Genetics, Genetic Tools

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to develop genetic and chemical tools that enable drug-inducible protein degradation in mouse tissues. These tools will facilitate more refined and insightful animal experiments on a range of topics, and improve our understanding of the mechanisms through which proteins are destroyed.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The function of proteins is normally studied by mutating DNA and observing the consequences. However, DNA mutations are slow to take effect, which makes it difficult to distinguish the immediate and direct consequences of protein removal from downstream effects. DNA mutations are also difficult to reverse, which would be desirable to understand how therapies might work. Targeted protein degradation solves this problem by allowing proteins to be directly targeted for destruction using small molecules. This works very quickly, and can be easily reversed once the small molecule is removed.

Drugs which work via a mechanism involving targeted protein degradation are being developed in large numbers by the pharmaceutical industry. This approach allows drugs to be developed against targets that were previously considered to be undruggable. In the longer term, our work will provide better systems to understand how these drugs work in tissues, which should help to make them more effective.

What outputs do you think you will see at the end of this project?

This work will lead to fundamental information on how protein degradation mechanisms vary between different types of cells within tissues. This information will help to improve the design of protein degrader drugs for use in human therapies, and to develop more effective methods to understand the function of proteins in mouse models of disease.

Who or what will benefit from these outputs, and how?

In the short-term, the outputs will benefit researchers using mouse models to understand normal ageing and disease by allowing them to obtain more useful information from a similar or smaller number of animals. In the longer term, they will benefit the pharmaceutical industry and patients who stand to benefit from protein degrader drugs.

Targeted protein degradation involves a suite of cutting edge technologies that are not currently well used by the mouse genetics community. The knowledge developed under this license will feed into services to be provided, under separate licenses, by transgenics facilities throughout the UK and overseas. This will allow the benefits of our work to be shared by the broader mouse genetics community.

How will you look to maximise the outputs of this work?

Through our involvement in the UK mouse genetics community we are already collaborating with leading laboratories in the UK that use mouse models to understand disease and develop better therapies. We will disseminate our findings through collaborations with these groups that showcase targeted protein degradation approaches, and by running a training course. We will also present our work at national and international meetings and publish datasets from our studies regardless of outcomes.

Species and numbers of animals expected to be used

- Mice: 7250

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The mouse is the most commonly used mammalian model organism. We aim to develop broadly applicable tools that improve how mice are used to understand human development and disease. All life stages will be used, from early embryos to ageing adults.

Typically, what will be done to an animal used in your project?

Animals used in this project will undergo administration of substances via injection, feeding, or surgically implanted drug minipumps, and be imaged under anaesthesia. In a small proportion of cases, these substances will cause cancer (e.g. colorectal cancer) to develop. Experimental durations will range from minutes to several weeks.

What are the expected impacts and/or adverse effects for the animals during your project?

Potential adverse effects include possible death under anaesthesia in survival surgeries, discomfort or pain following injections and surgical procedures, and toxicity induced during experiments to identify safe doses of novel drugs. Animals that develop colorectal tumours may experience rectal bleeding or diarrhoea (up to 24 hours), anaemia and weight loss.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Severities are considered to be moderate (~10% of procedures) or mild (~90% of procedures). For survival surgical procedures in juvenile animals, death from anaesthesia in and following surgery may occur more frequently (maximum 2% of procedures). Death rates occurring under anaesthesia in juvenile animals will be monitored and reported.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We aim to develop tools for use in mice, which need to be tested in the animal where they will ultimately be used. We also aim to understand mechanisms of protein degradation in different cell types, for which the mouse is well suited due to the similar cell type organisation of many tissues compared to human. For each target that we will explore, we will work with the subject experts in that area to ensure that they have embraced and exhausted the use of non-animal alternatives before we proceed to develop protein degradation methods to address a biological question.

There may be an opportunity in the future to explore other model organism such as flies in

order to understand the bio-distribution of a small molecule before moving into the mouse.

Which non-animal alternatives did you consider for use in this project?

Cell lines and ex vivo primary cell cultures.

Why were they not suitable?

The chemical substances used to trigger protein degradation can be rapidly broken down by the body, and often do not enter tissues such as the brain effectively. These processes are very challenging to model in vitro; the best way to understand how molecules behave in a living animal is to test them in that environment.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We estimate the required numbers of experimental animals based on our past experience, usage statistics of previous publications, as well as information provided from other license holders at our establishment. The numbers required for breeding, maintenance and production of experimental cohorts are derived from estimates provided by the animal facility. To stay updated on optimized experimental design, we will regularly seek guidance from biostatistical experts employed by our establishments.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We are developing transgenic lines that enable us to evaluate at least 5 different chemical genetic systems for targeted protein degradation (degron tags); some of these are in combination rather than developing separate transgenic lines for each system. For projects involving fusion of degron tags to endogenous mouse proteins, genetic constructs will always be tested in vitro in cultured cells to identify minimally disruptive fusion sites and reduce the instance of failure at the in vivo stage.

We will calculate the required number of matings to produce experimental cohorts based on the experience and breeding calculations used at the animal facility. Pilot studies will be performed to assist with planning for any experiments requiring large cohorts. We will refer to the PREPARE guidelines for study planning and organisation. All experiments will be reported according to the ARRIVE guidelines.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will implement efficient breeding procedures and will additionally perform well-designed pilot studies whenever possible to assess the potential for publishable results. We will additionally perform computational modelling and analysis of datasets to maximize

the information extracted from our datasets. Multiple tissues will be collected from each animal such that independent cohorts are not required to assess protein degradation in each tissue.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use mice as an animal model and determine which of several available methods for targeted protein degradation is most effective for different downstream experimental goals. These methods have previously been tested in mammals either minimally or not at all. Determining which methods cause the least harm is a critically important part of the assessment process, and will be a major output from this project.

Why can't you use animals that are less sentient?

Mice are the model organism of choice for studying a wide variety of normal and disease processes, and this project aims to develop better experimental approaches specifically for this species.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Determining which methods for targeted protein degradation have the lowest welfare cost is a critically important part of this project. We will implement pilot studies with increased monitoring the first time any novel substance is administered. Any studies involving surgical or invasive intervention will adopt appropriate pain management and post-operative care.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will stay up to date on best practice guidelines set forth and regularly updated from the NC3Rs website (Guidance on the Operations of ASPA - https://www.nc3rs.org.uk/).

We will follow the ARRIVE guidelines 2.0. For surgical procedures we follow the LASA guidelines on aseptic procedures (LASA 2017 - Guiding Principles for Preparing for and Undertaking Aseptic Surgery).

Routes and volumes for administration of substances are taken from Laboratory Animal Science Association good practice guidelines: administration of substances 1998 (http://www.procedureswithcare.org.uk/lasa_administration.pdf).
The animal facility has full AAALAC and ISO9001-2015 accreditation. To conform to these standards, we must work to a high level of quality control on all fronts including husbandry,

phenotyping and administrative processes.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay informed about the advances in the 3Rs by attending informational events provided locally and provided by the National Centre for the Replacement, Refinement and Reduction of Animals in Research. Our involvement in a national network with expertise in mouse genetics will also provide a route to discover new 3Rs approaches, and to disseminate information from our own advances in this area.

74. Molecular basis of infection-induced sickness behaviour

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Trypanosome, Pathogenesis, Virulence, Neuroinflammation, Behaviour

Animal types	Life stages
Mice	adult, juvenile, pregnant, neonate, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to understand how African trypanosomes, the causative agent of Human African trypanosomiasis or sleeping sickness, induce metabolic disease and neurological disorders affecting 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?

Human African trypanosomiasis is not only a devastating neglected tropical disease but can also be used as a tool to understand basic aspects of how our bodies combats infection. In our previous animal license, we generated significant knowledge regarding the mechanisms of skin colonisation, which is critical for parasite transmission. Additionally, we have generated a wealth of knowledge regarding the mechanisms underlying the

damage to the brain caused by the parasites, including potential drug interventions. Moving forward, our work will shed light on how the parasite causes weight loss and sleep disturbances. This knowledge is critical to understanding this complex disease and has the potential to open up new possibilities for the diagnosis and treatment of infectious diseases. More broadly it can also provide insights into how our bodies fight infections, and the unintended consequences these processes might have on normal health.

What outputs do you think you will see at the end of this project?

Reaching the goals of this project will not only increase the current knowledge available regarding trypanosome infections, in the longer term our findings could lead to improved chemotherapy or disease interventions. This would greatly benefit, both socially and economically, the developing countries where this disease is endemic. Beyond trypanosomiasis the results of this project could shed light on other infection or conditions that affect the host during systemic chronic infections, including systemic metabolism and brain function.

Who or what will benefit from these outputs, and how?

In the short term, our results will have an immediate impact in the scientific community, including those investigating parasitic infections, as well as those investigating weight loss, brain function, and behaviour in response to infection. In the longer term, we also anticipate that our work will have an impact on disease modelling and potentially improved intervention strategies, including developing novel therapies.

How will you look to maximise the outputs of this work?

Our work is collaborative and interdisciplinary, and so we anticipate that the outputs of this work will be maximised further through ongoing and future collaborations. We will engage with the scientific community in parasitology, immunology, and neurology to disseminate the knowledge generated from our work. Additionally, we will publish the work obtained from all the animal work conducted under this license (including negative data) to reduce the use of animals in duplicated work elsewhere.

Species and numbers of animals expected to be used

- Mice: 3,000

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 mice as they respond to infection in similar ways to humans and domestic animals such as cattle. Mice also have a well described immune system, genome and there is an array of mutant strains available, which will accelerate our research. The majority of trypanosome research studies have been based on this model and our results will be directly comparable to previous studies, maximising its impact. We will use adult mice (>6 weeks old) as their immune system will be fully formed and functional.

Typically, what will be done to an animal used in your project?

This project involves injecting mice with trypanosome parasites and monitoring how the infection progresses in the animal by routinely taking blood samples from the tail vein. Typically, wild-type mice will be infected for 1-3 weeks and may receive substances that can alter the function of the immune system via injection that may ameliorate the disease or shed light on the disease process. Mice will be closely monitored daily throughout the procedures and supportive treatments such as soft food will be provided, if necessary. Colonies of genetically altered mice will be bred and maintained, and then adult mice infected for 1-3 weeks via injection. Additionally, surgery may be performed to implant a wireless device to monitor electrical brain activity and skeletal muscle activity as a proxy to study sleep. In some instances, we will irradiate mice or treat with chemical compounds to modify the bone marrow, altering the immune system.

What are the expected impacts and/or adverse effects for the animals during your project?

In the majority of cases infection by trypanosomes may induce mild adverse effects such as the transient pain associate with injection or periodic mild clinical effects of the infection, such as the development of a rough / stary coat or lethargy. In some cases, the mice could develop more moderate clinical signs including staggering when walking. If no signs of clinical improvement are noted during this period, the animals will be humanely killed. It is also possible that a few animals may decline considerably becoming moribund, if this does occur the animals will be humanely killed. In rare instances, immunosuppression may occur as a result from irradiation. This could result in unintended side effects such as opportunistic infections. In such rare events, animals will be humanely killed to avoid unnecessary suffering.

Extensive experience of this infection model and a familiarity with the techniques used, allows us to recognise quickly any unexpected adverse signs. All mice will be closely monitored (at least every three days) throughout the procedures and recorded on the animal sheets and endpoints implemented as required. All animals will be humanely killed on completion of the experimental procedure.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

mice: mild 20%
mice: moderate 80%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Trypanosomes can be maintained in culture flasks and this technique will be used to create genetically modified parasite lines to study genes that may influence parasite survival and in initial drug investigations. However, trypanosome infection cause changes in multiple interconnected systems and organs within the host and neither the development of the disease nor the ultimate success or failure of potentially useful treatment strategies can be investigated fully in the isolation of a culture flask. For example, when the effects of trypanosomes were examined using an artificial model of the blood-brain barrier grown on a petri dish, no lasting damage to the integrity of the barrier was detected. When this was investigated using a mouse model of trypanosome infection, a progressive increase in barrier impairment was associated with disease development. This clearly illustrates a disparity between the mechanisms at play in tissue culture models and animal models of this disease.

Which non-animal alternatives did you consider for use in this project?

Human tissue obtained from stem cells generated on a dish (known as organoids) that could be employed in very limited circumstances. We have been developing in vitro culture systems to model migration of the parasites through body barriers such as the vasculature, and have established collaborations with other groups to validate the use of human skin explants generated in vitro to model how parasites invade tissues and dissemination to different organs. We have also explored the use of stem cell-derived brain organoids as a model to replace the use of live animals, with promising results. We will strive to employ these systems where possible.

Why were they not suitable?

Although these models are promising, as they stand, they do not fully recapitulate the responses obtained in animals. For example, brain organoids are devoid of innate immune cells, which limits its utility in the context of modelling infections in living animals. We are and will continue to invest efforts to keep developing organoids into more suitable culture models.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Mice: During the 5-year licence period requested it is estimated that a maximum of 3,000 mice will used.

Analyses of the data gained from the experiment will usually involve multiple conditions (e.g., uninfected and infected animals that are either treated with a drug or with a placebo) to ensure that the greatest amount of information is achieved from a small number of animals. In general, the number of mice required to achieve the goals set out in this project licence has been calculated using an experimental group size of 6-7. Additional statistical

support will be available when required to ensure that we gain the most accurate data using the minimal number of animals.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Working closely with a biostatistician, I have used data gathered over the past five years to estimate the number of animals required to achieve the expected level of significance for the most common experiments to be carried out under this license. The final estimates reflect the minimum number of animals required to answer the experimental questions behind this project license.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will conduct pilot experiments to determine appropriate sample size and power calculations. We are also building a tissue bank repository in the lab, obtained from previous experiments, that can be used as an alternative to conducting repeated experiments. Our breeding system ensures that littermates are used either in experiments or as breeders. As a general rule, we will maintain our colonies in low numbers and will only expand it when needed, and only for a finite period of time. At all times, we will follow the NC3Rs PREPARE guidelines and Norecopa guidelines.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The mouse is the most suitable laboratory animal to use in this system as trypanosome infections can be manipulated to reproduce each of the disease phases that are essentially similar to those found in both human and animal trypanosomiasis. In addition, the mouse provides a standardised animal with a wide range of analysis reagents and genetically altered research lines available. When generating bone marrow chimeras, where possible, we will use chemical compounds such as Busulfan as alternative, less aggressive, methods to irradiation in order to manipulate the immune system.

Extensive experience in the use of this mouse model provides familiarity of handling and maintaining infections and in the consistent induction of the various stages of the disease. This also results in a reduction in the severity of the procedures performed and in the number of animals required to achieve the goals of the experiment. To minimise the impact to welfare all animals will be closely monitored throughout the procedures and, if necessary, for those animals that require it, supportive treatments, such as soft food will be placed within easy reach.

Why can’t you use animals that are less sentient?

Trypanosomes are able to infect many vertebrate hosts, including mammals and fish. Whilst it is possible to infect zebrafish with trypanosomes, this requires the development of intricate infrastructure and husbandry of large fish such as carp to maintain the life cycle of the parasite. Further to this, it remains unclear whether zebrafish can be used to model the disease pathologies experienced by infected humans. Due to the extended nature of rodent models of infection, it is not possible to limit infection to an immature life stage. Moreover, most humans are infected during late adolescence or adulthood, when they have a mature immune system, meaning that juvenile rodents would not accurately reflect human disease pathology.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have and will continue to adopt NC3Rs and local guidelines to handling methods, housing conditions, and environmental enrichment, to ensure the welfare of the animals in our license. Our procedural approaches have been refined over the years to ensure that volume size, frequency of inoculation, and routes of administration (including needle size) do not cause distress to our animals. We typically monitor the animals after procedures to ensure there are not unwanted procedural side effects. We do not require analgesia but will maintain an open dialogue with the local veterinary team to make sure this is incorporate in a timely manner if needed.

Animal monitoring and pain management are an important part of all the procedures within the project. The need to minimise suffering is always considered when planning experiments and we routinely revise our experiments to reduce animal suffering. When using irradiation which can cause side effects in the gut, we will develop a more targeted approach thus improving the welfare of the animals, for example, by using alternative chemical reagents with less unwanted side effects (e.g., Busulfan). Of note, none of our experiments exceed a moderate severity level.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will ensure our experiments are designed in accordance with the National Centre for the Replacement, Refinement, and Reduction of Animals in Research (NC3Rs). Similarly, we will follow published best practice, eg Joint Working Group on Refinement.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will engage with the local ethical committee to ensure the work conducted under this license aligns to ethical standards, maintaining the 3Rs principles at the core of our activities. To this end, we will participate in national meetings organised by the establishment and National Centre for the Replacement, Refinement, and Reduction of Animals in Research (NC3Rs) in addition to in-house 3Rs meetings and events, to incorporate changes to our procedures in an effective and timely manner.

75. Optimising protective measures for cardiopulmonary bypass surgery

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

Surgery, Heart, Coronary, Bypass

Animal types	Life stages
Pigs	neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To evaluate ways of enhance the protection of the heart of children’s during cardiac bypass surgery.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

About one baby in every hundred is born with a defective heart. Many of these babies will have to undergo heart surgery to correct the defect, often whilst they are still very young. During surgery, it is often necessary to stop the heart temporarily to enable the surgeon to

repair the defect. Throughout this period, the blood supply to the baby's vital organs is maintained by coronary bypass. During the time while the heart is stopped, and in the period immediately after it is restarted, the heart can sustain damage that can permanently restrict its function. The techniques currently used to limit this damage are based on those developed for adult patients however, these are less effective in children.

Therefore, there is a need to develop more effective ways of protecting the heart of children's during heart surgery. The aim of the outlined work is to evaluate the effectiveness of interventions intended to protect the heart during coronary bypass surgery.

What outputs do you think you will see at the end of this project?

The primary output of the study will be new data relating to the mechanisms by which infant heart tissues sustain damage during coronary bypass surgery. In addition, the study will produce data relevant to the assessment of interventions aimed at minimising heart tissue injury during surgery. The data generated will be presented at conferences and published in peer-reviewed journals. In addition, the data will be used in applications for 'first in human' trials for interventions shown to improve the protection of the heart during coronary bypass surgery.

Who or what will benefit from these outputs, and how?

In the short term, the data generated will primarily benefit scientists and clinicians working to advance understanding of the cellular and molecular mechanisms responsible for heart tissue injury during coronary bypass surgery and to identify strategies to mitigate this. In the medium term, it is to be expected that the data will be used to facilitate the translation of improved interventions into 'first in human' clinical trials. In the long term, the work will potentially benefit the many thousands of babies and children who each year undergo cardiac surgery to correct life threatening heart defects.

How will you look to maximise the outputs of this work?

The data will be published, including any negative results, in open access and peer reviewed scientific journals, in addition to presentations at international conferences. Following the successful translation of improved interventions, it is to be expected that these will become standardised across the NHS and other health care providers.

Species and numbers of animals expected to be used

- Pigs: 328

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 pigs have been selected for these studies because their anatomy, size, physiology and state of development is very similar to that of babies and young children, thereby enabling the clinical scenario to be closely replicated.

Typically, what will be done to an animal used in your project?

The outlined work will involve three types of experiment:

Protocol 1) Studies to investigate the biological cause of tissue injury during coronary bypass surgery and to assess interventions aimed at preventing this, will be performed under terminal anaesthesia at the end of which the animal will be killed without recovery. Young pigs will be anaesthetised and prepared for coronary bypass. The heart will be stopped for a representative time period, and the animal maintained on bypass until the heart is restarted. Prior to stopping the heart, drugs with an action likely to protect the heart from injury may be given. Throughout the study period, which may extend for up to three hours after the heart is restarted, small blood and heart tissue samples will be collected for subsequent analysis.

Protocol 2) To investigate the mechanisms by which a pre-existing heart injury (representative of that of many babies undergoing surgery) increases the susceptibility to injury during coronary bypass surgery, a minor heart injury will be induced by the temporary occlusion of a coronary blood vessel under general anaesthesia. Blood vessel occlusion will be achieved using either a balloon catheter, inserted into a peripheral blood vessel and guided into place with the aid of non-invasive imaging or by the temporary placement of a surgical ligature. Pigs undergoing these procedures are expected to make an uneventful recovery and to resume normal behaviour within a few hours. Around one month later the pigs will be used in a terminal study as outlined under protocol 1) above.

Protocol 3) Studies to determine the benefits of drug treatment shown to improve protection of the heart in the short term (studies 1 & 2 above) will be further assessed using the same procedure as described under protocol 1) above, but with animals continuing on study for up to 72 hours, either maintained under general anaesthesia or in a conscious state following recovery. Pigs allowed to recover will be given pain killers in line with that given to babies that have undergone heart surgery. At the end of the test period, the pigs will be anaesthetised and killed to enable tissue samples to be collected for subsequent analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

Roughly 40% of animals will be used in protocol 1), as described above, and will experience no more than mild transient stress associated with the induction of anaesthesia.

About 40% of animals will be used under protocol 2), as described above. Animals used in this protocol may experience mild pain upon recovery from anaesthesia however, this will be controlled through the use of pain killers and all animals are expected to resume normal behaviour within 24 hours of surgery. There is a small risk that some animals may develop signs of heart failure before they enter the second 'terminal' phase of the study however, any such development would be gradual in onset and the animals would be killed as soon as signs developed and before any overt suffering occurred.

The majority of animals used under protocol 3), as described above, will be maintained under anaesthesia without recovery and so will experience no more than the mild transient stress associated with the induction of anaesthesia. A small proportion of animals may be

permitted to recover and so may experience mild pain however, this will be controlled through the use of pain killers.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

50% Non-recovery

50% Moderate

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The primary aim of the study is to identify interventions that will improve the clinical outcome of babies who have to undergo corrective heart surgery during which the heart is stopped and restarted. It is not possible to replicate this situation using either isolated organ, tissues culture or by computer simulation. Furthermore, it will not be possible to translate the findings into the clinical setting without first demonstrating their safety and effectiveness in a representative animal model. Consequently, it is not possible to conduct this study without using animals.

Which non-animal alternatives did you consider for use in this project?

We have done as much preliminary work as possible using data from patient derived tissue taken for clinical analysis. However, it is not possible to move this research forwards without the use of an animal models.

Why were they not suitable?

There are no alternatives to the use of animals for the outlined studies and it is not possible to jump straight to humans with this research without first producing animal based data to support the proposed interventions.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 data from previous studies of this type. All studies will be carefully designed to use the smallest number of animals needed to obtain meaningful results (observation of reduced cardiac damage and improved cardiac function in some groups compared to others).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We thought of ways to reduce the number of treatment groups, for example if we test two interventions we should be able to only generate one control group, which we can use for both. The project is planned in a sequential manner so that only interventions that are successful in early phases of the study will be progressed into the main study. Study designs will be checked using the NC3R's Experimental Design Assistant. Variability will be reduced by the use of standardised protocols for the surgeons, perfusionists and anaesthetists; to keep variability low and thereby reduce the number of pigs required.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Pilot studies will be used to generate the data needed to optimise group size for the interventions undertaken. We will also bio-bank tissue collected at the end of studies for use by ourselves or collaborators, which may avoid the need for animals in some future studies.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Around 60% of the animals will be used in procedure performed under non-recovery anaesthesia, thereby limiting any suffering to that incurred during the induction of anaesthesia. Following anaesthetic induction, the animal will be placed on cardiac bypass, during and following which, cardioprotective treatments will be performed to assess their effectiveness at improving clinical outcomes

The remaining animals will undergo a procedure to induce a myocardium injury under recovery general anaesthesia. The methodology used for this has been refined during previous work and results in a lesion that does not cause overt clinical signs. Upon recovery, the animals will be given pain relief under the direction of a specialised veterinary anaesthetist. Surgery will be performed by specialist paediatric cardiac surgeons supported by a team with extensive experience in by-pass surgery. The animal care staff looking after the animal, all have extensive experience in their post-operative

care.

For young pigs, the ligation method will be used in preference to the balloon catheter technique for inducing a myocardial infarction. This is because the balloon catheter technique is difficult to perform in small pigs due to the size of the blood vessels involved. Approximately 4 weeks after the induction of the myocardial infarction, the animals will be used in a procedure as outlined above. A small subset of these animal may be allowed to recover following bypass and maintained alive for up to 72 hours in order to determine if cardioprotective treatment given to minimise inflammation in the immediate post- operative period are effective at improving clinical outcomes. Animals allowed to recover will be given drugs to control pain under the direction of a specialist veterinary anaesthetist.

Why can’t you use animals that are less sentient?

This study requires an animal that is anatomically and physiologically similar to humans and is of a size that enable heart surgery to be undertaken in a manner that closely replicates that performed on babies and young children, including the monitoring of all parameters normally recorded in a clinical setting. Pigs are the least sentient of the species suitable for the outlined work and have been used extensively as a translational model for human cardiovascular surgery including transplants and valve replacement. It is not possible to use less sentient animals because their physiology differs markedly from humans and therefore would not be acceptable to the regulators responsible for authorising first in human clinical trials.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Upon arrival in the unit the pigs will be habituated to humans by regular close contact and hand feeding them treats. The pigs will be group housed and provided with an enriched environment. To optimise welfare and avoid the stress associated with manual handling, the pigs will be trained to voluntarily enter the weighing crate and transport trolly. Anaesthesia will be provided by specialist veterinary anaesthetist, who will also be responsible for post operative pain control. Surgery will be performed by paediatric cardiovascular surgeons, supported by a team with extensive experience in performing by-pass surgery in pigs. The surgical techniques used have all be refined during previous studies. The animal care staff, who look after the pigs, are all experience in the provision of post- operative care.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All procedures will be conducted in accordance with LASA, NC3Rs Guidelines on best practice for surgery and any clinical guidelines for each substance administrated.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Through engagement in local and regional NC3Rs events, through information circulated by our Named Information Officer, through regular reviews of the literature and through feedback from members of my staff involved in my institute's Early Career Researchers 3R committee.

76. Pathogenesis of Osteoarthritis and related connective tissue disorders

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Osteoarthritis (OA), Marfan syndrome, Cartilage, Mechanoflammation, growth plate

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?

My group are interested in the diseases and normal physiology of the musculoskeletal system. Our work will seek to improve our understanding of the cellular and molecular processes relevant to the common degenerative disease osteoarthritis, as well as other less common conditions such as Marfan Syndrome.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 main disease that we focus on is osteoarthritis (OA) which is the biggest unmet

healthcare need in developed countries. There are currently no disease modifying drugs in this disease and these are urgently required. We are also interested in inherited conditions of the connective tissues which manifest with abnormal skeletal and joint problems, such as Marfan Syndrome. Musculoskeletal diseases of this sort are poorly understood and murine models have proved to be a meaningful way to unravel their biology and discover novel treatments.

What outputs do you think you will see at the end of this project?

We have already demonstrated that using an approach in which we combine human data, in vitro data and murine studies, we can discover novel treatments that can be tested in patients. Indeed, we have one such clinical study in progress that has arisen with help from preclinical studies. We expect to continue to use this approach to refine pathway knowledge and treatments better to ensure that only the best candidate drugs are tested in patients. As a clinician I am mindful of the patient being the primary focus of our work although there are earlier beneficiaries from a scientific perspective that will including publications and dissemination of science at international meetings.

Who or what will benefit from these outputs, and how?

Beneficiaries in the short to medium term include academic. Work that we lead has contributed substantially to a new body of literature surrounding the molecular pathways that drive OA and the importance of mechanical load in this process. This has contributed to the ‘rewriting’ the OA textbooks which means that the next generation of doctors understands this disease in a radical new way. This will also be the case for understanding why Marfan Syndrome patients develop scoliosis (curvature of the spine) and how and why exercise is important for the long-term health of the skeleton. We expect this PPL to continue to add to the body of publications and scientific dissemination to relevant international groups.

Ultimately, I expect the patient to benefit (medium to long term). Responses from patients to some of our basic research publications suggest strongly that they are excited by the new potential drugs identified by our work. They simply do not have anything available to them at the moment and largely suffer in silence. We are in a position to deliver small validation clinical studies in patients. If successful, these would lead to clinical trials with the hope that drug regulatory authorities would licence them for clinical use. The Federal Drugs Administration in the United States, and European Medicines Agency (Europe) are aware of the huge need for disease modifying drugs in OA. Once licenced, a new drug would completely change the landscape for patients, potentially reduce the need for surgical joint replacement, and have huge effects on improving the quality of life of individuals.

Moreover, our work will help to elucidate the benefits of exercise on the skeletal system particularly during early skeletal growth. This has important implications for how we encourage youngsters to exercise in adolescence to improve skeletal health as they age. Finally, beneficiaries include industry (medium to long term). There is money to be made by industry in these areas. For OA, studies are very expensive to run and to date most have failed. Drugs (especially repurposed ones) that target novel pathways discovered through basic research have not, on the whole, been tested yet. A successful trial arising from this approach is important to demonstrate that success is possible. It would provide industry with a much-needed boost of confidence that OA is a disease worthy of investment and that pre-clinical models have a valuable role in this pathway.

How will you look to maximise the outputs of this work?

We have a strong track record in publishing high quality papers and for ensuring that negative studies are also published. We generally have an open approach to data sharing at meetings to ensure rapid dissemination, and use open archive resources ahead of publication. We have a very collaborative approach to our research and frequently collaborate with colleagues, both in the immediate environment and also at other universities. This includes sharing unpublished data and sharing new expertise in models.

Species and numbers of animals expected to be used

- Mice: 23450

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 diseases such as OA, identifying patients with very early disease, and having access to their joint tissue is very difficult. This has significantly limited our ability to understand the earliest origins of disease, necessary for being able to understand the causes and key pathways. All animals (even fish) can get OA if they overuse their joints. Rodent models have the advantage of replicating the joint anatomy of bipedal mammals and therefore have best translatability. OA largely occurs in older people and animals but can occur prematurely in younger individuals who have sustained a destabilising joint injury e.g. meniscal tear (common amongst skiers and footballers). Subjecting young adult mice to surgical joint destabilision produces all the hallmarks of human OA (age-related and post-traumatic).

These models do have proven translatability, for example we identified nerve growth factor (NGF) as a key mediator of pain in murine OA. Anti-NGF was subsequently shown to be a powerful analgesic in human disease (albeit sadly not licenced by FDA due to adverse side effects). Mice are also amenable to genetic modification and creation of specific mutations that are found in patient disease. This is the case for MFS where a human point mutation leads to mice with highly characteristic features of human MFS.

Typically, what will be done to an animal used in your project?

Genetically altered mice with susceptibility to OA will either be bred in house or bought in. Some animals may be injected with tamoxifen to induce deletion of a specific gene in an inducible manner.

The majority of animals will be entered into OA studies. The mice would undergo joint destabilising surgery (taking approximately 15 mins) under inhalation anaesthesia. Analgesia would be given in the immediate peri-operative period, as any human would be after a surgical procedure.

Rather than inducing OA we may want to make a small cut in the cartilage in order to see how well it heals. This is a cartilage healing model rather than an osteoarthritis model, but the two may relate to one another. This is performed under general anaesthetic once to

the cartilage behind the knee cap. Post operative analgesia is given in the immediate post operative period.

Pain behaviour may be tested as this is the most important clinical feature of human disease. Typically this would be performed weekly by incapacitance testing (measuring how much weight is being taken by the operated compared with non-operated limb). Other pain assessments might also be performed. These are not regarded as harmful to the mouse.

Some experiments may be done in germ free mice in which there are no bacterial at all in the animal (including gut and skin). Mice tend to be a little smaller but are otherwise similar to those that are not germ free.

Occasionally it might be necessary to immobilise the joint to assess how this changes disease. Joint immobilisation may be by cutting the sciatic nerve on one side (under anaesthesia) which renders the limb both paralysed and numb (no sensation). Cutting the sciatic nerve at the same time as the femoral nerve leads to the mouse dragging the leg behind them. This procedure is done infrequently and only for short durations. An alternative strategy that we have explored is putting a 3D printed plastic splint over the knee joint of the mouse. These are removable and are well tolerated.

Some mice may require imaging which is done in a microCT scanner under general anaesthesia. It is not thought to cause harm to the mice.

Regular drug treatments up to daily for 28 days may be given over this time. Mice would be killed humanely, typically at 4 or 8 weeks post-surgery (maximum 20 weeks).

Some of our experiments look at tissue injury responses “ex vivo” i.e. after fresh removal from a recently live animal. For these the animal, that may be genetically modified, is humanely killed and fresh tissue (e.g. skin, ear cartilage, hip cartilage) removed.

What are the expected impacts and/or adverse effects for the animals during your project?

Generally our procedures are very well tolerated and are not, in our hands, associated with weight loss or ill-health. An expected adverse effect in our OA models would be immediate post operative pain (for which analgesia is administered) and the development of pain as OA progresses. This starts quite late (from 6-8 weeks post surgery) and worsens over time. It is a subtle finding, which we demonstrated by showing that some methods used to pick up pain in inflammatory arthritis models are not sensitive enough to detect pain behaviour in our OA surgical model.

If joint immobilisation is introduced (either by cutting the nerves that supply the leg or by splinting), this can alter the gait of the animals. Cutting the sciatic nerve alone causes the mice to walk with a straight leg (because they lack hamstring power and can’t bend the knee). Although the gait is abnormal, they have normal levels of activity. Combining sciatic and femoral nerve injuries leads to the mouse dragging the leg behind them and no weight is borne through this limb. This can sometimes cause sores to develop on the dragged part of the foot, so we avoid doing this for longer than a couple of weeks. Splinting appears to be well tolerated and is reversible. Each of these has a distinct value in our experiments. Splinting is the most refined of our models but it needs to be replaced with a new splint weekly under anaesthesia as the mouse grows. It also does not offer the

'weightlessness' that double neurectomy offers.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild severity: 50%

Moderate severity: 50%

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

There are several reasons why human OA is a challenging disease to study. These include the fact that the disease is a slowly progressive one and there are many influences over time that contribute to how varied it is in patients. Thus end stage human OA tissue samples rarely resemble one another either macroscopically (when looking at it) or when studying the molecules in it. A second point is that the disease is often without symptoms at early stages. In other words, the patient doesn’t know they have a problem until the joint is significantly worn down. This makes study of early disease almost impossible in human subjects. Finally, acquiring samples of normal joint tissues from individuals is highly challenging. This is partly because it is considered unethical to remove cartilage from a healthy joint as the biopsy may itself induce OA. Normal or early diseased tissue acquisition e.g. from post mortem samples is very challenging.

For these reasons it is essential to have a model in which early disease can be studied, the genetic and environmental factors can be controlled and where we can use genetic modification in order to study the function of specific molecules in the disease course. It is also possible to perform our experiments 'germ free' (completely lacking all gut and skin bacteria) – the mouse being the only mammal where this is currently possible.

Which non-animal alternatives did you consider for use in this project?

Our OA pathogenesis programme uses a combination of in vitro (laboratory), ex vivo (analysis of human surgical tissues), and in vivo (mouse) techniques with validation using patients where possible. This helps us to prioritise only the best targets to take through to mouse studies. Our group are also trying to develop better three dimensional systems that recapitulate human tissue in vitro. In time it is hoped that these will help with this prioritisation also and reduce in vivo use.

Why were they not suitable?

At present it is very hard to model the unique mechanical environment in the joint/growth plate and to model the multiple tissues that contribute to the biology both in health and in disease.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

This is based on our use as a group over the past 5 years (using previous home office returns) and takes into consideration our anticipated continuation of the programme.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Our guiding principle is to reduce mouse numbers by ensuring that our studies are of high quality and provide us with a result that we can trust and is meaningful from a patient perspective. The OA surgical models have 100% penetrance in male mice. That is to say all male mice will get robust disease within a few weeks of surgery. Almost all of our studies that have looked at OA severity by joint histology (tissue sections) are performed in male mice and in strains that exhibit high levels of disease. Rather than discard female mice we try to use these for other experiments such as skin wounding and ear punch studies. To improve robustness of experimental design we randomize mice to treatment group and always ensure that scoring of disease outcomes is blinded. We perform power calculations (using on-line tools) prior to experimental set up and consult biostatistical colleagues. For surgical OA, we tend to look at just two time points post surgery (4 and 8 weeks). We continually review and revise our experimental procedures to reduce animal numbers. For instance we have previously used ear biopsy injury (as the ear contains cartilage) as a surrogate for joint cartilage injury models. The murine model of MFS has 100% penetrance and is evident in both male and female mice. Preliminary data have allowed us to calculate accurately, the number of mice needed 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?

Breeding colonies will be managed in line with the best practice guidelines which includes weekly review. Particular attention will be paid to genetic stability and good breeding performance. Genotyping is confirmed by a commercial provider in an efficient manner. 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. Strains that are not in use are routinely cryopreserved.

Recently we have acquired a Slide Scanner which will be used to acquire high throughput digital images of murine joints. This will speed up delivery of results and should improve image quality, both of which should help us to reduce numbers of mice used in experiments.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

OA models. Surgical models of OA produce robust disease within a few weeks of surgery and it produces disease similar to that seen in humans. Generally, we favour using models induced by surgically destabilisation of the joint (by cutting the meniscus). These models are generally less inflammatory than those induced by chemicals so they cause less pain. Removal of the meniscus increases the severity of surgically induced OA leading to earlier disease. Pain behaviour also develops earlier after removal of the meniscus but we have no evidence that the severity of pain differs between the surgical OA models. The mouse is amenable to genetic modification, which forms an important part of this programme.

Marfan syndrome model. Mice harbouring a genetic mutation commonly seen in humans with the disease have been generated and are commercially available. These mice have 100% penetrance in both males and females and display both the cardiovascular features as well as musculoskeletal features of human disease.

Germ free mice. We are interested to know whether the gut microbiome (the bugs in the gut) could influence the course of OA in our models. If we find that there are changes in how germ free mice respond to joint injury, we would extend these studies to looking at other injuries (e.g. of skin) in germ free and gut microbiome reconstituted animals. An important complementary approach will be to use broad spectrum antibiotic therapy in mice caged in our standard facility to ‘clear’ the gut microbiome, rather than using germ free mice.

Joint immobilisation. We know that immobilisation of the joint can prevent the development of OA and halt the progression of established disease. Several methods exist for joint immobilisation including neurectomy, where the nerves that supply the muscles of the leg are cut. There are two main nerves to the leg - the sciatic nerve and the femoral nerve. Single neurectomies (sciatic or femoral) are well tolerated by the mice: femoral neurectomy causes a very subtle foot drop resulting in a slightly exaggerated ‘slapping gait’; sciatic neurectomy causes the mice to walk with a fully extended (straight) knee. This causes a slightly unusual looking gait but activity levels are maintained. Double neurectomy leads to complete paralysis of the leg and the mice drag the leg behind them. This can cause sores to develop on the dragged part of the foot. Double neurectomy is only used for a small number of experiments and typically for no more than 2 weeks in total (although we have performed experiments for as long as 12 weeks in the past without ill effect). As a refinement, we have developed 3D printed external splints that can be applied to the mouse hind limb. The splint immobilises the knee but leaves the hip and ankle free. The splint is thin (< 0.75 mm) and light (< 0.8g). Using external splinting reduces the need for a further operation (no neurectomy procedure required), is reversible

and reduces the likelihood of ulceration and discomfort from paralysis of the limb. This method of joint immobilisation is adaptable (i.e. it is possible to change the material used to splint, change the shape or fastening mechanisms of the splint) and can be reapplied easily over the course of an experiment if required. The disadvantage is that it has to be changed at least weekly as the mouse grows. We have little experience using this beyond 1 week thus far.

Increasing joint use. Complete joint immobilisation causes thinning (atrophy) of the articular cartilage, but an alternative way to study the role of mechanical load is to increase load. We have done this by giving mice access to voluntary exercise wheels in the cage. There are no adverse events associated with this.

Pain Assessments. We use a range of pain tests which are well validated by others and by ourselves although mostly we use Incapacitance testing which is well tolerated and performs well in our hands. It is one of the least disruptive behaviour tests as it simply measures the amount of weight that the mice put through their two hind limbs when they are in a semi-upright position in the apparatus. Making sure the assessor is blinded is essential and this improves the robustness of the experiment outcome.

In vivo imaging. This allows imaging to be performed in live mice over time and has the potential to change the primary outcome measure from tissue analysis to early imaging. Mainly (<90%) this would be non-invasive micro CT imaging, but scans might be enhanced by injection of contrast agents. This could reduce the time of experiments (and numbers) and provide a sensitive means to screen different genetically modified animals.

Why can’t you use animals that are less sentient?

OA is a disease, whose main risk factor (after mechanical stress) is ageing. The young joint has a remarkable ability to repair itself and this dramatically reduces once skeletal maturity has occurred. Studies, therefore, in immature joints would distort the normal biological response and not be relevant to disease. It has been possible to do some experiments in fish. Fish also develop evidence of some of the bone aspects of OA in their spines, but these joints do not resemble human joints and miss many of the tissue components.

MFS can not be reproduced in other disease models to my knowledge.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Our model refinement is being reviewed by us in a continuous manner. This goes for the surgical protocols as well as how we measure the outcomes. An example of this includes shifting away from the slower surgical models of OA to slightly faster and more robust ones (partial meniscectomy, PMX).

Where pregnant females are treated with tamoxifen by oral gavage, the mice will be culled after weaning of litters.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Surgical procedures are performed in line with LASA guidelines. We comply with ARRIVE

guidelines and for most of the journals that we publish in, the ARRIVE checklist is required with submission. Our surgical models are in accordance with the published international OA society gold standard.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The team will regularly attend University, Departmental and Institute 'NC3R' and Animal Welfare Meetings to keep abreast of developments in the area. The PPL holder chairs the animal user group at the Institute through which new developments are also disseminated. We will also have access to a NC3R’s regional manager and will liaise with the establishments Named Information Officer.

77. Regulation of cell fate allocation in the developing mouse embryo

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Embryo development, Stem cells, Cell differentiation, Gene regulation

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We aim to provide new information about the nature of the genetic pathways that control the processes of early mammalian embryo development within the mothers womb. We will use a combination of pre- existing and newly generated strains of genetically modified mice to address this aim.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 goals are to continue to gain new information about the development of the early embryo, and how it interacts with the uterus during implantation and pregnancy. Importantly we want to identify the key proteins that regulate the process of normal embryo development and direct the naïve early cells of the embryo to form all the different cells types including for example blood and heart muscle that make up the large number of different organs of the body. Growth and normal development of the embryo depends on

how it interacts with the uterine tissue of the mother. Placental development requires collaboration of embryonic and maternal cells in order to build a fully functional organ within the uterus necessary to exchange vital nutrients and gases between the mother and the baby. Our studies also aim to provide a more detailed understanding about the cells that make and maintain this intimate connection necessary to sustain the pregnancy. Understanding the pathways that normally control the behaviour of cells in the embryo has many applications including understanding the basis of spontaneous failure to maintain pregnancy (in the human population between 8-20% of pregnancies result in miscarriage before 20 weeks of gestation), the causes of congenital defects like heart defects (affecting 1% of live born babies) and spina bifida (affecting 1 in 2,500 live births), as well providing insights into how these pathways go awry in the adult to result in diseases such as cancers.

What outputs do you think you will see at the end of this project?

Publications in leading Developmental Biology and basic science journals.

Generation of genomewide datasets (gene expression studies on a tissue and single cell level) that will be made publically available to the scientific community via appropriate on line repositories.

Identification of essential transcriptional regulators and new modes of gene regulation operating in the early mammalian embryo.

Who or what will benefit from these outputs, and how?

Our experimental programme aims to provide new information to the worldwide developmental biology community via publication of peer reviewed scientific publications. On average we aim to publish 2-3 papers per year, and to maximise the impact of our research outputs we deposit pre-prints of our papers on the bioarchives BioRXiv web portal, making these freely available to the public before publication. Our ability to correlate the effects of making genetic alterations on the ability of the embryo to grow, develop normally and form the organs of the body, help inform the medical community about the possible genetic causes underlying birth defects in the human population. Our in-depth genetic and biochemical analyses also serve to help the research programmes of other basic science groups who are studying the processes which allow the cells of our bodies to form the highly diverse tissue types (for example muscle, bone, nerves, blood) that make up the organ systems of the body.

How will you look to maximise the outputs of this work?

Our group maintains a high degree of collaborations with local, UK and worldwide colleagues. These collaborations seek to provide a more time and cost efficient mechanism for addressing basic aspects of cell and developmental biology and minimise duplicating our efforts. We routinely present our unpublished data and observations at work-in-progress sessions (both on-line and in person), seminars and at national and international conferences. We make our reagents (cell lines, tissues, gene probes), in vitro protocols and genetically altered mouse lines freely available on request to other researchers.

Species and numbers of animals expected to be used

- Mice: 35800

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice and humans diverged from a common ancestor about 140 million years ago, yet most salient aspects of mammalian physiology have not diverged significantly between these animals during this time. Comparative studies of the completed genome sequences of mice and humans show they share approximately the same number of genes. Indeed, comparative anatomical, embryological and physiological studies have shown that the mouse share the same basic organ systems, skeleton, reproductive cycles to humans. These similarities validate the use of the mouse as an excellent model organism for studying human developmental biology. The feature that distinguishes the mouse from other mammals as a model system is its genetic tractability, and it is now very straightforward to make precise changes in genes in the mouse using a variety of molecular technologies. We use embryonic stem cells and zygotes to introduce genetic changes into the mouse genome and generate novel genetically altered mouse strains. We then assess the impact of these changes on the ability of these modified mouse embryos to develop normally after they implant into the uterus of the mother, during the first half of the pregnancy.

Typically, what will be done to an animal used in your project?

Typically the genetically altered mouse strains are maintained by routine breeding with normal wild type mates and the resulting offspring screened using a small ear tissue biopsy. Carrier animals of the desired genetic makeup are then mated and the females humanely killed on specific days of pregnancy and the embryos recovered from the uterus for study in the laboratory. A fraction of animals will experience mild, transient pain and no lasting harm from administration of substances by injection using standard routes (intravenous, subcutaneous, intraperitoneal). For the generation of new genetically altered strains animals will experience some discomfort after surgery and some mild pain which will be treated with analgesics.

What are the expected impacts and/or adverse effects for the animals during your project?

Mice will have minor "key hole" surgery to implant embryos into the reproductive tracts (female mice), or to render male mice infertile by vasectomy. They are expected to recover quickly and will be given painkillers and closely monitored post-operatively. Mice will experience very mild transient pain from the administration of substances by injection using standard routes (intravenous, subcutaneous, intraperitoneal).

Expected severity categories and the 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 96% sub-threshold, 1% mild, 3% moderate

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Our research is aimed at understanding the development of the mammalian embryo and how the intact growing embryo interacts with the uterus of the mother. We need to use mice to model these processes as it is not possible to manipulate the genome or perform in depth embryo studies in humans. Correct development of the embryo in time and space is critically dependent on the environment within which it occurs and this cannot be accurately modelled by tissue culture models.

Which non-animal alternatives did you consider for use in this project?

Where appropriate we make extensive use of cell culture systems to validate and further study cellular and molecular phenotypes. For example we make extensive use of protocols using genetically modified embryonic cells to discover whether the genetic changes prevent them from forming different cell types such as, for example, muscle and blood in the dish. We also use 3D culture model systems including tissue "organoids" that mimic certain aspects of organogenesis. These in vitro protocols are combined with state-of-the art gene profiling (RNA-sequencing) and chromatin analyses (DNA accessibility-sequencing; Chromatin-sequencing). Recent papers have described the generation of so-termed "synthetic" mouse embryos that can be generated in tissue culture chambers by mixing cell lines derived from the pre-implantation embryonic and extra-embryonic populations of the early embryo.

Why were they not suitable?

Post-implantation embryonic development is highly complex and cannot be fully recapitulated using the in vitro systems currently available to us. The reported efficiency of generating synthetic embryos that superficially resemble early in vivo recovered embryos is exceptionally low (less than 1%) and as such these models have limited value for studying the orderly growth and tissue interactions that underlie development of the intact embryo in vivo. Thus this is not an accurate alternative for modelling the multitude of cell and tissue interactions occurring at the embryonic-maternal interface necessary to sustain in utero 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?

Our experiments typically involve the generation and analysis of embryonic lethal and tissue specific mutations. Working together with the experienced technical staff in our facility, and building on my 35 years of active research using laboratory mice for embryological and molecular biological experiments, we have optimized our breeding programme to ensure that we generate the minimum number of animals necessary for the successful outcome and publication of our research projects. The numbers proposed are based on this previous experience and from gauging the numbers of embryos we expect to collect from each pregnant mouse. The genotype ratios are calculated assuming Mendelian rules of inheritance. For the estimating the number of animals to be used in this project we have been guided by our previously submitted annual Home Office returns.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The reproducibility of scientific data has come under increasing focus in the last few years with many of the top journals requiring the inclusion of rigorous experimental details. In designing our experiments together with my research team members, and in consultation with our local bioinformatics colleagues, we first review the relevant literature and discuss the experimental approach. We quality control all of our reagents (e.g. antibodies) in cell lines and transfected cell lines to optimize protocols (e.g. fixation conditions). To obtain statistically significant data we prepare RNA or chromatin from age matched and where indicated sex-matched biological replicates (i.e. 2 independent samples of mutant and wild type tissue pools), and assay technical triplicates (i.e. three identical samples of each genotype are profiled).

We are very proficient at generating new strains of genetically altered mice, and where appropriate obtain strains from scientific colleagues or from one of the International mouse repositories.

Recently we have incorporated into our experimental strategies a newly developed technology that involves the use of so-termed "degron-tagged" genes. Through the addition of a small molecular tag to an endogenous gene (using CRISP-R technology) the resulting protein can be degraded in vivo within 2 hours via the administration of a small molecule to the pregnant mouse. Using animals carrying both copies of the degron tagged gene we can generate 100% of embryos in which the tagged protein has been deleted (as opposed to 25% using a conventional genetic cross of two heterozygous animals).

All of our experiments involving genetically altered mouse strains comply with the ARRIVE guidelines (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?

Breeding colonies will be managed in line with the best practice guidelines. Particular attention will be paid to genetic stability and breeding performance. Data from breeding animals are readily available from our in-house data bases 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. We are careful to control

the genetic background of the mice to the best of our ability by routinely back-crossing genetic alterations onto the C57BL6/J background to minimize genetic variability.

Strains not actively in use have been archived as targeted ES cell lines, or frozen sperm within a dedicated liquid nitrogen facilities within our lab. We make all of our genetically altered strains freely available to the scientific community.

We are careful to optimise any new protocols (e.g. design of CRISP-R reagents, validation of antibody reagents) using readily available tissue and cell lines before incorporating embryonic material into our experiments.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We work entirely on the laboratory mouse. The development of very sophisticated methodologies for the temporal and spatial control of endogenous gene function make mice an extremely tractable model organism for deciphering the mammalian genome. Due to the high level of genetic conservation between the mouse and human genomes, understanding gene function in the mouse embryo provides important insights into the roles played by their homologs in humans.

For the generation of new genetically altered mouse strains it is necessary to perform two surgical procedures both of which will be carried out on animals following induction of anaesthesia by the route and agents appropriate for the nature and duration of the procedure. For surgical transfer of oocytes and blastocysts we perform key-hole abdominal surgery to expose the oviducts (necessary for oocyte transfer) or uterine horns (blastocyst transfer). For vasectomy we will render the males sterile via a single scrotal incision allowing exposure of the left and right vas deferens which are sealed via cautery.

We consider injection or oral gavage of gene altering substances such as for example tamoxifen as the most refined approach, rather than via addition to the drinking water, since our experiments require precise dose and timing of substance administration in order to coincide with specific gestational stages of embryo development.

Why can’t you use animals that are less sentient?

Non-mammalian animals cannot be used for our experiments studying early post-implantation mammalian development. Mammalian development is unique in that it requires that the embryo implant within the uterine environment and establish connections with the maternal in order for development to proceed.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Our colonies of mice are housed in biosecure cage systems, and colonies subjected to regular health screens to ensure the animals are living under pathogen free conditions that could otherwise affect their general health and well being. All cages are checked on a daily basis by the facility staff, who alert my group members to any perceived issues affecting individual animals so that we can act accordingly to ensure the animals are correctly monitored. Any animals displaying signs of obvious distress (e.g. hunched posture, lethargy, rectal or cervical prolapse) are killed immediately. Animals subjected to minor surgical procedures are administered peri-operative analgesia and closely monitored over the next 48 hours for signs of continued pain requiring additional analgesic administration.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the guidelines published by NC3R's, LASA, ARRIVE and PREPARE to ensure that our experiments are carried out in the most refined way.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

As members of our host organization, we have access to a NC3R regional manager and internal 3Rs meetings are regularly scheduled that my group members and myself attend. We also receive via email regular NC3R newsletters keeping us up-to-date with new developments. We have access to our Host Institution Named Information Officer with whom we can consult to ensure we are following best practice for our experiments. The host Institution also has regular Animal Welfare meetings where any new guidance is provided to the Project and Personal licence holders. We also access the NC3R website (www.nc3rs.org.uk).

78. Regulation of immunity by unconventional T cells

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Unconventional T cells, infection, inflammation, cancer

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?

Our research aims to understand how, where and when immune cells are activated and the consequences for health when immune responses are dysregulated.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 mechanisms and signals controlling immune cell activation and function is critical to design novel vaccination and immuno-therapeutic strategies to protect from disease. Thus, the questions addressed in our studies are clinically relevant and the mechanisms identified in our studies will be validated with samples from patients with inflammatory (e.g. inflammatory bowel disease), infectious diseases (e.g viral or bacterial infections) and cancer. The data generated by this project not only will help our basic understanding of immune cell biology but also it will inform further research to exploit the targeted manipulation of specific immune cell subsets for therapy of various disorders.

What outputs do you think you will see at the end of this project?

We expect this project will generate new knowledge and information on the ways in which our immune system fights infections and cancer and how it is dysregulated in inflammatory disorders. Building on this new knowledge, we also expect to identify novel targets for immunotherapy of infections, inflammatory diseases and cancer, that can be tested in preclinical models and, eventually, in clinical trials. The results arising from this project will be made accessible to academics by publishing them in peer-reviewed journals and presenting them in national and international meetings. Results will be also made accessible to the general public by using social networks, web stories, podcasts, videos and/or panel discussions/debates held for the media and general public.

Who or what will benefit from these outputs, and how?

While our work is basic research, it has strong translational potential, due to the fact that all disease settings we work on closely mirror relevant clinical situations of high public health impact. In the short term the information generated in this project will benefit the UK and international scientific communities. This is a state-of-the-art multidisciplinary project which will represent a significant step forward on our understanding of the processes and signals controlling the activation and function of immune cells. We anticipate that these results will be of interest for scientists working on a broad range of disciplines including immunology, but also virology, microbiology or respiratory medicine. In the medium to long-term we expect our results will provide proof-of concept data supporting the targeted manipulation of immune responses for the treatment of infections, auto-immune diseases or cancer.

This may result in the development of new treatments and/or repurpose of available interventions.

How will you look to maximise the outputs of this work?

We will maximise the outputs of this work through already established collaborations with clinicians and scientists in the UK and abroad. We will also collaborate with pharmaceutical companies and perform outreach work collaborating with charities and patient groups. New knowledge will be disseminated in publications and conferences as well as more broadly to reach the general public, patient groups and clinicians by scientific seminars, press releases, radio and TV interviews, blogs, social media, outreach activities such as school visits or the pint of science, and others.

Species and numbers of animals expected to be used

- Mice: 8400

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The mouse is the ideal organism for these investigations since: (a) it is the longstanding choice of immunologists and has identified the operating principles of the immune system on which major clinical advances are based, (b) there is an immense spectrum of reagents available and (c) is a powerful genetic tool for assignment of function to particular genes

and molecules.

For our studies we will use young and adult mice, to ensure we are using animals with a fully developed immune system.

Typically, what will be done to an animal used in your project?

Typically, we will compare immune responses between WT and genetically altered mice or between mice treated with a substance (e.g. inhibitors) vs mock-treated mice. In all our experiments, the duration of the experiments will vary depending on the model and will consider many features of the disease specific to the experiment. We always aim to maintain the duration of experiments to the minimum required to address the scientific need.

For infection: Animals will be infected with a defined dose of a known pathogen strain (e.g bacteria such as Klebsiella pneumonia), and either killed at a pre-determined time point to assess tissue damage, as well as specific immune parameters (e.g. immune cell activation) that we consider relevant for severity and correlate with disease progression, or mice will be monitored over the whole course of infection for clinical signs and weight loss to determine severity of the infection. All infections we study are self-limited and are resolved within a maximum of three weeks unless animals reach humane end points before. Where pre-determined time points are chosen, we always aim to maintain duration of experiments to the minimum required to address the scientific need and obtain meaningful scientific data. For instance, some immune cells are activated very early in the immune response, hence analyses can be performed at early time-points after infection (1-2 days). Other immune responses (e.g. antibody secretion) require longer periods of time (several weeks) hence longer experiments are required.

For intestinal damage/inflammation: Animals will receive a treatment to induce intestinal inflammation (e.g., dextran sodium sulfate (DSS) in the drinking water). They may be treated with drugs or antibodies by injection (weekly, or more regularly, as required) to test immune pathways and targets of resistance or susceptibility to progression of disease. We are interested as to how the changes by drugs, antibodies, diet, immune modulators, genetic alterations in the mice, will impact the host response during intestinal inflammation and its response to therapy. The animals will have their disease state monitored over time (typically over a week) using symptoms such as weight loss or diarrhoea.

For cancer models: Animals will be injected with tumour cells (which may be labelled), to study how immune cells regulate tumour progression as well as how tumours affect immune cell functions.

Several models of tumours will be used which may be superficial (such as breast cancer) requiring intradermal injection of tumour cells; or internal (such as ovarian cancer) requiring intraperitoneal or intravenous injection of cells. Tumour progression can be monitored by imaging at different times after injection. Tumour growth may be slow, or it may be rapid. Knowledge of the tumour models used will allow a good prediction over the time frame of the disease progression. Mice will be likely killed on the basis of a time period (typically up to two months) rather than based on their clinical signs. Therefore, an experimental end point (time controlled) will most likely occur before a humane end point (as determined by deterioration of heath conditions).

What are the expected impacts and/or adverse effects for the animals during your

project?

For some experiments we will inject immune cells or substances such as blocking antibodies which can modify the immune response. These are expected to cause no or minimal side effects. In mouse models of respiratory infections or intestinal inflammation, mice may suffer flu-like and colitis-like symptoms respectively, including weight loss, hypothermia, laboured breathing, diarrhoea, hunched position, lack of movement, all to varying degrees. For all models, we have detailed knowledge of the days of highest disease severity and will monitor mice closely these days. Once these critical days are overcome, mice recover rapidly. Only a minority of mice will undergo a full-time course of infection to link interventions or genetic ablation to changes in severity, while the vast majority of mice will be killed at predetermined time points to study lung damage and immune parameters. For tumour models, for most animals, we do not expect to see anything more than subtle changes in the clinical condition or behaviour. In some cases, internal tumour growth could result in abdominal bloating, weight loss and signs of poor condition (hunching, piloerection, inactivity).

We have knowledge and experience of the models that we use and will seek additional expert advice if needed from our collaborators or experts in the field. We are mostly able to predict well the time frame over which the animals will not show adverse effects for each of our models. The use of certain interventions with molecules of the immune response, or antibodies directed against them, can make this slightly less predictable, but the animals are monitored closely in this case to minimise any suffering and we aim to terminate experiments before the onset of potential adverse effects and humanely kill mice before they reach the humane endpoint.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severities predicted by this proposal are subthreshold (20%) mild (50%) and moderate (30%).

Adoptive transfer of cells or injection of immuno-modulatory substances (such as inhibitors or blocking antibodies) are not expected to cause more than mild symptoms. In models of infection or intestinal damage/inflammation we expect the majority of the mice to experience only mild symptoms, and only a small percentage may reach moderate severity. Typically, the level of severity will depend on the time- points at which immune responses will be analysed post infection/inflammation or on the dose of the infectious agent. The mildest possible protocol will be used to mimic human disease. For tumour models we expect that the vast majority of the animals will only experience mild symptoms, although moderate symptoms may be reached in some of the mice

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The core of our studies is to define the molecular basis for the interactions of different cell types within intact tissues, in response to challenges to those organs. Such complex, dynamic responses cannot be achieved by use solely of isolated cells, and cannot be adequately represented ex vivo by use of cell lines or mathematical models. For instance, resident immune populations (the key targets of this work) are shaped by the complex interplay of tissue-specific signals and stromal cell types within different organs, that cannot be effectively recapitulated in vitro. Whilst investigations in cell lines have been extremely useful to define signalling pathways involved in immune cell activation, as cell lines have been modified they will not truly represent the process of immune cell activation under physiological conditions. Thus, animal experimentation is necessary for accurate characterisation of these processes and to validate findings from the cell lines.

Which non-animal alternatives did you consider for use in this project?

We have recently optimised in the lab models of long-term expansion of epithelial organoids that we culture with immune cells to study the immune-epithelial crosstalk. Cells used for organoids could be isolated from humans or the experimental mouse models and could help to replace as well as reduce the number of mice used. In these organoid-immune cell co-culture systems we can identify how specific signals derived from immune cells (e.g. cytokines) contribute to the regulation of intestinal homeostasis, and to repair the intestine after damage. We are also exploring the use of cancer- derived organoids which we can culture in vitro with immune cells to understand the tumour-immune cell crosstalk. We have also developed in vitro infection models using cell lines, primary murine cells or human PBMCs. For instance, we can infect in vitro human PBMCs with bacteria or virus (e.g.
Klebsiella pneumonia or influenza) and explore how the infection regulates the activation and function of specific immune cell populations.

We closely follow the development of even more complex cell culture systems (called “organ-on-a- chip), but so far they are not able to mirror all the interactions between many different cell types in the living organism. We are collaborating with clinicians to use samples from patients to validate that our findings are clinically relevant and to reduce the usage of mice

Why were they not suitable?

While in vitro experiments provide very valuable mechanistic information about the processes mediating immune cell activation in vitro, they are not able to mirror all the interactions between many different cell types in living tissues. Culture systems cannot reproduce the high complexity of immune responses that are dependent on cell movement from the bone marrow through the blood into infected organs, under the influence of systemic factors such as hormones, microbiota or the central nervous system. It is also impossible to fully reproduce lung or gut tissue, composed of a great variety of different cells on a complex extracellular matrix. For instance, in vitro cell culture cannot capture the complexities of the physiological immune response to infection, since after infection, for example of the lung by aerosol, cells in the lung become activated and then in turn activate immune cells that traffic to the lymph nodes become further activated and multiply and

then return to the lung to kill the infectious agent. It is not possible to model these complex multi-organ events in the in vitro assays. Thus, the possibility to replace animal experiments by in vitro experiments is so far limited to specific questions. However, we actively pursue further development of in vitro systems in the lab to extend their potential and reduce the use of animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals to be used has been estimated on the basis of the previous 10 years of animal work of my lab and the current landscape of projects of the lab going forward, staffing in my lab, the current funding and the planned funding applications.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

For the design of mouse experiments, we follow the recommendations as outlined in the PREPARE guidelines (https://norecopa.no/PREPARE0) and the NC3R's Experimental Design Assistant. We employ several strategies to try to reduce the number of mice in the study including:

We will always aim to maximise the amount of data (pathology information and gene expression) we get from each mouse, for example by obtaining multiple tissues or experimental read-outs from the same mouse.

We routinely perform experiments on both males and females, to make full use of the mouse colony and to avoid sex-bias in our results.

We try to include several conditions (e.g., injection of substances such as different blocking antibodies) in separate treatment groups in the same experiment, to save on untreated controls and make full use of multifactorial design to enhance statistical power.

We use(d) statistics and past experiments, including pilot experiments, to define the minimum number of mice required for statistical significance to obtain robust reproducible data that determines outcome. Using the above online tools, power calculations will be performed to determine minimal treatment group size for maximal statistical information.

Whenever possible, we will limit the use of genetic models (that often require many generations breeding) by treating the mice with chemical agents or antibodies to either block immune-system components or enhance them.

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 minimise the number of animals by mostly using inbred mouse strains, and by

housing them under identical conditions to limit variability.
We shall investigate the ready availability of genetically-altered strains from the community, prior to generating any de novo.

We will avoid overbreeding, and lines under sporadic use will be maintained at low levels, and frozen whenever practicable, and/or maintained in collaboration with other licences to minimise redundant breeding.

We store many organs from experimental mice in case novel findings or future research directions require analysis of these organs.

We will perform pilot experiments to test the doses of the products we are going to inject as well as to estimate the effect sizes mentioned above.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The mouse is the ideal organism for these investigations since: (1) it is the longstanding choice of immunologists and has successfully identified the key operating principles of the immune system on which major clinical advances are based, (2) there is an immense spectrum of reagents available, and (3) the mouse has emerged as an extraordinarily powerful genetic organism, permitting the generation and analysis of genetically modified strains that permit assignment of function to particular genes and molecules.

Genetically altered mouse strains which will be used in this study include: (a) knock out mice that lack particular effector molecules or cell types of the immune system, (b) transgenic mice that offer the opportunity to study particular aspect of responses that otherwise could not be monitored due to the low frequency of antigen-specific cells (e.g. T cell receptor-transgenic mice) and (c) reporter mice that make it possible to study particular cellular processes overcoming the suboptimal detection threshold of alternative assays. The vast majority of mice bred under our protocols are not expected to exhibit any adverse phenotype. Nonetheless, mice will be maintained in IVCs (individually ventilated cages) under barrier environment, to avoid infections.

All mouse experiments performed will be designed such that the minimum severity in terms of infection or inflammation burden required to show effects will be employed. In most of our experiments of infection and inflammation the measure of immune parameters is performed at very early time-points before mice develop any measurable adverse effects. Nonetheless, in a minority of experiments mice will need to go through the full induction of disease and recover from it. Our experiments are set up in a way that all mice recover from the disease and recuperate normal weight gain. Mice are carefully monitored during disease induction, and all measures are taken to minimise pain and discomfort (including provision of wet food in the cage, additional bedding, recovery racks).

Why can’t you use animals that are less sentient?

While some immune responses can be studied in less sentient animals such as flies or fish, the immune cells our studies focus on (MAIT or NKT cells) are not present in these species. However, they are present and conserved in mice and humans.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We always aim to refine our procedures. We actively share refinement and improvements in techniques and seek to constantly improve our models to ensure that we are minimising any harm to the animals, as this also helps to improve the accuracy of our study and reduce artefacts caused by stress. For instance, tunnel handling will be rolled out within the next years, which causes less anxiety than traditional tail handling. We are guided by and seek advice from local NVS policy on non-invasive procedures, as well as improved pain management, to minimise harm.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Unless otherwise specified, the work in this project will be designed using the principles outlined in the PREPARE guidelines for planning animal research and testing (2017) and in the LASA Guiding Principles on good practice for Animal Welfare and Ethical Review Bodies. We will also follow LASA Guidelines on administration of substances.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We are regularly updated within the institute on advances in the 3Rs from NC3Rs (https://www.nc3rs.org.uk/nc3rs-newsletters) and NORECOPA, and we actively seek information on possible improvements in discussions with colleagues and collaborators at in-house meetings and external conferences. The NC3R Regional Project Manager keeps us informed on key advances and knowledge. As experts in the field we are up-to-date with all the latest publications as well as the latest research from conferences and collaborations, thus we are fully aware of any refinements published, that could help us in refining our experimental mouse models further. We constantly interrogate the literature to refine our experimental mouse models and test potential targets relevant to human disease. Whenever we are able to refine techniques without impacting the scientific validity of our work, we aim to implement advances, for example enriched environments for mice to reduce stress levels and avoid overgrooming.

79. Revascularisation after ischaemia for treatment of cardiovascular 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

Angiogenesis, Cardiovascular disease, Diabetes, Vascular Endothelial Growth Factor, Splicing

Animal types	Life stages
Rats	neonate, juvenile, adult, pregnant, embryo
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?

To determine whether altering expression of specific splice forms of VEGF can restore vascularisation in animal models of cardiovascular 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?

Cardiovascular disease is still the primary cause of mortality and death in the UK, and the

most common forms of cardiovascular disease are caused by an inability to recover blood flow after an ischemic insult to a tissue (e.g. the heart, limbs or brain). The lack of blood flow recovery in many cases, particularly in diabetes and in obesity, is due to expression of endogenous anti-angiogenic growth factors such as VEGF-A_{165}b, controlled by activation of signal transduction pathways in monocytes and/or muscle cells. If we can find out ways of switching off these anti-angiogenic factors, for instance using neutralising antibodies or inhibitors of the signalling pathways, we could develop treatments for people with cardiovascular disease, preventing death due to heart attacks and stroke or loss of limbs due to peripheral arterial disease.

What outputs do you think you will see at the end of this project?

We expect by the end of this project to see the development of new potential therapeutics, either antibodies or small molecules, that are being taken through the clinical trial process for treatment of people with cardiovascular disease. It will also result in publication of scientific data supporting the principle that endogenous inhibition of revascularisation is a major contributory factor for cardiovascular disease, which will stimulate additional approaches beyond those outlined here, by biotech, pharma and academia.

Who or what will benefit from these outputs, and how?

The ultimate beneficiaries of these outputs will be the 50% of people who develop cardiovascular disease. Additional beneficiaries will be the UK economy as these findings are transferred to the biotech or pharma industry, and clinicians who will be able to more easily manage their patients through medical rather than surgical intervention and at an earlier stage.

How will you look to maximise the outputs of this work?

We will publish the data generated, collaborate with other groups both in academia and in industry who may be looking to develop new approaches to cardiovascular disease, and present the results at scientific conferences.

Species and numbers of animals expected to be used

- Mice: 1500
- Rats: 100

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice and rats are used as there are models in these animals that closely mimic human cardiovascular disease, particularly in the case of diabetic animals such as those genetically lacking obesity control, lacking insulin or insulin receptor function, or that have been exposed to a diet that induces diabetes. Rats are used as they have pharmacokinetic and pharmacodynamic properties that are closer to humans than mice.

Typically, what will be done to an animal used in your project?

Animals are bred or allowed to express their phenotype that reflects lack of revascularisation either due to transgenic expression of specific traits (e.g, monocyte specific expression of Wnt5a or selective knockout of splicing factors) or become obese and/or diabetic (e.g. ob/ob mice, ZDF rats) or are fed a diet that induces diabetes (e.g. high fat high sucrose diet). Vasculopathy may be non-invasively determined by urine collection and/or sensory testing for neuropathy by stimulation with an escapable non-noxious (von Frey hair) or mildly noxious (high temperature from a light) stimuli and measuring paw withdrawal. They are then subjected to non-invasive imaging of blood flow to the paw, followed by induction of ischemia by surgical ligation of one of the arteries supplying the leg (e.g. femoral) under anaesthesia. After closure of the surgical wound, and re-imaging they are allowed to recover, and then at intervals (e.g. 3 days, 7 days, etc up to five times) are anaesthetised and blood flow to the paw imaged non invasively again. After 3-4 weeks the animals are terminally anaesthetised and killed by perfusion fixation or overdose and tissues removed for analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

Some postoperative pain is likely and is treated with analgesics. The loss of blood flow in the short term to the foot can lead to lameness, and in more severe instances foot necrosis and self amputation. The post-operative pain is usually temporary- for a day or two. It is possible that the ischemia can lead to ulceration or necrosis in some animals. The animals will be closely monitored for this and would be killed if it develops.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The vast majority of animals will experience moderately severity – a surgical procedure under anaesthetic and post-surgical recovery. Some animals will only be used for breeding.

80% Moderate

20% Mild (breeding 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?

Collateralisation is by definition an in vivo event. The remodelling depends on multiple cell

types (e.g. endothelial, pericyte, haematopoietic, smooth muscle, fibroblasts, immune cells), embedded within biological matrices (e.g. basement membrane, interstitium, glycocalyx), exposed to forces that are imposed on them by the animal (e.g. oxygen tension, blood flow, mechanical and shear forces). To understand the molecular mechanisms underlying this vascular remodelling we make use of the best available evidence from isolated systems (e.g. endothelial cells in culture, human white cells isolated from patients with disease) to develop testable hypothesis that are likely to explain the physiological change, but to test those hypotheses it is necessary to perform experiments in the system in which it operates, i.e. the intact blood vessel, in as close to a representative environment of that seen in vivo. That means carrying out experiments on animals.

Which non-animal alternatives did you consider for use in this project?

In vitro experiments using immune cells, and endothelial-monocyte cell co-culture are undertaken to initially test the hypothesis but there is no non animal alternative to determine recovery from ischemia as this is by definition an in vivo process.

Why were they not suitable?

Co-culture experiments do not take into account the influence of circulation, muscle movement and lymphatic effects as well as blood flow and haemodynamics.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 undertake 10 sets of mouse experiments in the first two years, and 5 per year after that. Each set will use 20 animals (10 control and 10 treated). We will undertake 1 set of rat experiments each year. This is based on funded projects for the next two years, and estimated funded projects after the first two years. We estimate it will take an equal number of mice to generate the transgenic animals for the experiments as for the experiments as we are often using complex genetics (e.g. tissue specific inducible knockouts combined with tissue specific over-expressors), so half of the animals used in protocol 2 will also be used in protocol 1.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We use successive imaging of animals and normalise blood flow to the non operated side so that intra animal variation can be used. This reduces the number of animals significantly. Historical controls and block design are also used to reduce the animals per group, and we use both male and female animals to ensure that results can be applied to both sexes.

What measures, apart from good experimental design, will you use to optimise the

number of animals you plan to use in your project?

We use breeding pairs to generate transgenic mice with the fewest numbers of animals required to produce the animals for the studies. We generally do not undertake pilot studies as we are not generally using agents that have never been tried in mice before, unless they are variants of antibodies, which are not expected to have any unpredictable adverse events. This means we can undertake the minimum number of studies required rather than undertaking a pilot study and then having to repeat it with a larger number of animals. At the end of the experiments we collect as many tissues as possible as well as blood and monocytes from the spleen and these are stored in blocks for subsequent analysis and histology.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 because they are the animal with the least neurophysiological sensitivity in which parameters can be compared across literature studies, and in which these models have been optimised and are most representative of the human diseases that they model.

In addition, genetically modified and harmful mutant mice may be used. For example, transgenic animals with inducible and tissue specific promoters. These are used to help identify specific molecules important in vascular remodelling.

In addition, a second rodent species will be used to determine relevance beyond mice. Rats are the most appropriate species as they display impaired blood flow after ligation in the ZDF model compared with non diabetic rats, and the mechanisms are likely to be the same as in the mice. A second species is part of the criteria for clinical development of a therapeutic.

Harmful mutant animals with relevant disease susceptibility (e.g. db/db mice, ob/ob mice, ZDF rats) may be used to investigate the influence of these disease states on collateralisation. As illustrated previously, the choice of the most appropriate disease model results in the best replication of the human disease process, with the least harm and suffering caused to the minimum number of animals, and in which processes can be studied in a quantitative and reproducible fashion to reduce the number of animals required in each study, using published, validated and refined experimental procedures to enhance comparison with other reports of collateralisation. The high fat, and genetic models provide a defined onset of early diabetic vasculopathy. The HFHS model of diabetes can also be induced in a transgenic animal in which a molecule hypothesised to modify the collateralisation process has been manipulated. In this way, the role of that molecule in the diabetes-related vascular remodelling process can be examined. This means we can determine in fewer mice earlier whether this is a key part of the pathway before going on to cross the transgenic animal with harmful mutant models of diabetes

(e.g. db/db mice), which would require many more animals and an imprecise disease onset. These animals will be monitored according to the Generic Animal Welfare score sheet to minimize severity and identify minimal severity endpoint.

The most refined models of human disease will be adopted. For example, the highly-refined and widely-accepted high fat high sucrose model of type II diabetes (as published by the AMDCC) and the Ins2-/- Akita mouse will be adopted. This enables robust induction of vessel remodelling in diabetes coupled with minimised adverse events of the diabetic state, and also maximises the number of published studies with which our results can be compared. Even in these refined models, however, adverse effects of diabetes induction still occur (particularly polydypsia, polyuria, and weight loss).

These adverse events are inherent in the development of the diabetic state via these methods.

The use of sensory testing and urine collection allows us to determine a level of vasculopathy non invasively that allows us to refine the models further. The increase in urine albumin is a sign of vascular disease that has progressed to the point of pathological impairment of normal vascular function. The sensory threshold reduction, also a sensitive technique to identify vasculopathy, indicates that vascular function is modified - for mechanical withdrawal thresholds an indication of altered sensory nerve function, and for heat withdrawal threshold an indication of altered central mechanisms - a slightly later effect. Thus these three outputs allow us to sensitively monitor different stages of vascular dysfunction in diabetes and metabolic syndrome.

Surgical procedures are conducted with aseptic precautions according to LASA principles. Animals are treated with analgesics after surgery. These all minimize animal suffering. On some occasions, a multiple series of interventions may be made on individual animals (e.g. ischemia, sensory testing, urine collection, imaging). Only one of these is moderate and the others are mild severity for each individual technique. The combination of techniques enables collateralisation to be investigated in animals with a validated diabetic vascular phenotype, refining the models further.

Why can’t you use animals that are less sentient?

Impaired revascularisation is not apparent in immature animals, and is less or not apparent in less sentient animals as regenerative capabilities are greater in non mammalian species. Revascularisation after ischemia takes days to weeks, so recovery experiments are required.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will use post-operative pain management and monitor the animals for signs of necrosis in the foot daily. If the necrosis worsens and continues beyond 21 days animals will be killed. All technicians and researchers are trained in assessing animals for condition, for diabetic complications, and to act accordingly. Cages are changed more regularly due to the polyuria in these animals. The induction of diabetes does not result in severe hypo or hyperglycaemia resulting in a refinement compared with chemical ablation of islets (e.g. use of streptozotocin).

What published best practice guidance will you follow to ensure experiments are

conducted in the most refined way?

AMDCC (Animal Models of Diabetic Complications Consortium) and LASA for animal care and models. ARRIVE Guidelines for reporting (and publishing) animal experiments, and PREPARE guidelines for experimental design. Guidelines for dose volumes and frequency are taken from the IQ Consortium 3Rs leadership groups position paper.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We have regular training, and present our work at conferences where latest developments are presented and discussed, including refinements, replacement technologies and methods to reduce animal use. Funding for conferences is included on all submitted grant applications.

80. Role of cellular senescence in normal physiology and disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Cancer, Paediatric, Brain, Lung, Pituitary

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 main goal of this project is to understand the function of senescent cells in normal biology and in disease, with a particular focus on cancer. Through this basic research, we aim to identify novel therapies and to translate these discoveries into humans.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Cancer is one of the main causes of premature death in humans. In the UK, there are around 375,000 new cases of cancer every year, but only around 50% of cancer patients will survive 10 years after diagnosis. Cancer is mostly an ageing-related disease, and incidence has been growing for the last several decades in parallel with the increase in human life span. Studies of large human populations estimate that 1 in 2 people will

develop cancer in their lifetime.

In this proposal, we aim to understand better how cancer is initiated, and how it grows and propagates with the ultimate goal of identifying vulnerabilities that can be used to develop new efficacious anti- cancer therapies. In particular, we are very interested in studying the role that particular cells, called senescent cells, play at different stages of cancer development.

Cancers contain cells that can multiply faster than normal (cancer cells), and other cells that do not multiply at all (senescent cells). Research carried out over the last 5 years, including in my own group, has shown that senescent cells play a key role in the way cancers start, grow and spread. Senescent cells drive cancer development through the production and release of potent/strong chemicals that fuel growth of neighbouring cancer cells.

Senescent cells also play a role in cancer relapse (when the cancer comes back after therapy). Current anti-cancer treatments, e.g., radiotherapy and chemotherapy, target dividing cells but have little effect on senescent cells. This means that senescent cells can persist in the cancer bed, continuing to release key stimulating factors, and so continuing to promote further growth and division in neighbouring cells, causing cancer relapse. Researchers are beginning to investigate whether killing senescent as well as dividing cancer cells could lead to more effective cancer treatments.

This project aims to study senescent cells, what they do to promote cancer initiation, growth and relapse, and importantly, how to eliminate these senescent cells using novel, selective drugs to complement current anti-cancer therapies.

What outputs do you think you will see at the end of this project?

Some of the benefits are:

Increased knowledge of the effects of cellular senescence in cancer, including both adult and childhood cancers. This may provide insights into the processes underlying the initial stages of tumourigenesis (i.e., the process of forming cancer), which are generally not explored in the cancer field. Likewise, we will reveal insights into the mechanisms leading to cancer relapse and identify complementary approaches to reduce relapse.

Identification of novel anti-cancer treatments. This proposal will test several new therapies in preclinical models, revealing potential new treatments against these tumours, which could be further assessed in clinical trials or small human studies with our clinical collaborators. Through our previous licence, we have identified and tested the efficacy of novel treatments against a clinically aggressive childhood pituitary tumour.

Generation of novel mouse models for the benefit of the research community. The mouse models generated through this research will impact the field of senescence beyond the scope of this proposal. For instance, researchers in the fields of ageing and age-related diseases will be interested in using these genetic tools in their studies. The mouse models will be made available subject to MTA (Material Transfer Agreement) approval. We have an excellent track record in generation and sharing of genetically altered (GA) mouse models.

The findings of our research will be disseminated in open access research publications,

conferences, workshops, patients' groups and charities, among others. The licence holder has a strong record in publications and is invited routinely to present his research in national and international conferences.

Who or what will benefit from these outputs, and how?

The beneficiaries of this project are:

The research community. Through the publications and research presentations, researchers in a variety of fields will benefit, including researchers working in cancer and cell biology, developmental biology and ageing, among other basic disciplines. Likewise, clinical colleagues such as oncologists and endocrinologists interested in developing new clinical trials will benefit from this research as it will provide novel therapies that could be beneficial for their patients. Likewise, drug developers in academia or industry and pharmaceutical companies will benefit from this research, for example building up on the findings to drive new drug development.

The patients will benefit from this research, as our ultimate goal is to identify novel treatments for the benefit of the patients. The treatments identified through this research will be tested in preclinical platforms, for example, cancer cell lines, pieces of tumours grown in the laboratory and mouse models. If proven to be efficacious against particular cancer or tumour types, this evidence will be used to initiate clinical trials. I collaborate very closely with oncologists and neurosurgeons and have already been involved in the development of two clinical trials.

How will you look to maximise the outputs of this work?

Sharing data, reagents and mouse models and communicating our progress to scientific, clinical, industry and lay audiences, and ensuring that this information is freely available, form the basis for maximising the output of this work. We will communicate our work to academic researchers, clinician scientists, healthcare practitioners, drug developers and pharmaceutical companies. This will ensure translation of novel diagnostic tools and experimental therapies to the clinic, so that patients' lives improve.

As previously mentioned, the results from this research will be published in open access journals and presented in conferences world-wide. Results obtained from research are not always positive and realistically, some experiments may yield negative results. Nonetheless, negative results can often be very informative and helpful for the research community. Communication of negative results is particularly important when the research involves animal experiments, because this is likely to avoid duplication. In my group, we have previously published negative results in research journals and we will continue to do so.

The proposed project will generate a significant amount of data obtained from cells harvested from tumours and cancers in mice. Molecular data will be deposited to publicly available online databases. Molecular biology reagents enabling the generation of tumour models in mice will be made available publicly as well, for dissemination to the wider tumour research community. Murine cancer cell lines generated during the course of this study will be deposited to publicly accessible cell repositories and databanks. New mouse strains will be available to the research community, as we have previously done.

I have established strategic collaborations both nationally and internationally with basic

researchers as well as clinical colleagues to maximise the impact of the research. This is demonstrated through the wide range of colleagues included in the research publications from my lab. Additionally, I have established industrial collaborations with pharmaceutical companies to facilitate the development of clinical trials in the patients.

Finally, I believe strongly in public engagement and science communication, and we will participate in public seminars throughout the course of this project.

Species and numbers of animals expected to be used

- Mice: Mice 30000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The purpose of this proposal is to understand the role of senescence in normal physiology (e.g., embryonic development) and disease (e.g., cancer) with a focus in paediatric brain and pituitary tumours, as well as lung cancer. Mice are essential for the proposed research. Cells in a Petri dish cannot fully replicate the diversity and complex biology of the mammalian organs. Moreover, invertebrate or non-mammalian models cannot reproduce many biological and physiological aspects of human tumours. Mice share a similar genetics, development and anatomy with humans, and brain, pituitary and lung cancer models in mice display many similarities to the human counterparts. Therefore, to make clinically relevant discoveries and develop more effective therapies for human cancers, experimental work using mice is essential. To achieve this goal, we will use all stages from early embryonic development to adulthood and aged mice.

Embryos: It is well established that cancer cells reactivate mechanisms that are important during normal embryonic development. This is particularly true in the case of paediatric cancers, which normally are initiated in the fetus. Paediatric cancers are developmental disorders and as such, their study within the developing embryo is warranted if we aim to understand the mechanisms underlying cancer initiation and growth. For example, brain and pituitary can develop in a sustainable way only within the context of the developing embryo and no in vitro system can mimic these conditions. Hence, an understanding of normal development, and of disturbed development that leads to birth defects (including cancer), requires analysis of whole animal embryos.

Adult mice: Although initial stages of cancer formation can usually be detected embryonically, the development of a proper cancer (e.g., with a large cell mass) requires a latency period of postnatal life. In this proposal we need to study these stages of cancer development, in addition to the early stages. An important reason is that cancer patients are normally diagnosed with advanced cancer, therefore we need to recapitulate these stages in mice if we aim to identify efficacious treatments. In addition, some of the experiments that we will conduct in this project will require immunosuppressed adult mice, for example to generate patient-derived xenograft (PDX) models in which human tumour cells are transplanted into a particular organ of an adult mouse. Once transplanted, tumour cells need a period of weeks to months to develop a tumour.

Aged mice: Senescent cells naturally accumulate in tissues and organs during ageing in mice and humans. These senescent cells can contribute to tumour initiation and expansion through the secretion of potent chemicals. Therefore, we need to investigate the function of senescent cells in aged mice and compare these factors with those produced in younger mice.

Typically, what will be done to an animal used in your project?

Mice are kept in a facility that is fully dedicated to maintain mice for research. Mice are usually grouped (except males that have been used as stud males, as they will fight if put back together with other males) in individually ventilated cages. They have permanent access to food and water, and are kept are at optimum temperature and humidity. Their environment is enriched with bedding, nesting material, refuges, etc. We will perform different types of procedures:

Breeding of genetically-modified mouse models (GEMMs). We have either developed ourselves at UCL or acquired from collaborators and suppliers a number of GEMMs that we use for our research. Usually, the genetic alterations do not manifest during breeding in the vast majority of the mice. This is because they are not activated. Therefore, the adverse effects of the breeders are usually sub- threshold or mild. These breeders are used to generate offspring, which will be used for experiments. We get a small biopsy through an ear notch, which only causes transient pain, to isolate DNA and genotype the offspring (genotyping is to assess whether the genetic alteration of interest is present in the mouse), so that we can maintain only the right GEMMs. Mice are normally bred for around 1 year. The number of mice we generate during the 5-year licence is around 22000.

Manipulation of embryos in utero:

Sometimes we need to activate genetic alterations in embryos when developing in utero, for example by administrating activating drugs (e.g., tamoxifen). In this experiments, pregnant females are injected with the drug to activate a genetic alteration in the embryo. Usually, embryos develop well and are born normally. Likewise, females rarely have any complications. The number of pregnant females subjected to this procedure is less than 100 per year and the total number of embryos, less than 500 per year.

We will also need to electroporate embryos or inject viruses to activate the expression of mutant genes that may cause cancer or disease, or activate the expression of a gene that will facilitate monitoring tumour development. We usually use this approach to generate a mouse model of paediatric high-grade glioma (gliomas are tumours that derive from a type of neural cell in the brain called glial cell). Pregnant females are anaesthetised and the uteri exposed through a small incision in the abdominal cavity. Embryos (usually at day 12 or 13 of gestation) are visualised through the uterus wall and injected with substances or electroporated. For electroporation, we inject DNA in the brain cavity and then electrical pulses are passed between electrodes placed across the uterus, either side of the embryo. After the procedure, the uterus in placed in the abdominal cavity and the incision is sutured. Pregnant females recover very well, and complications are very rare. Embryos usually are delivered normally and up to 95% of injected/electroporated embryos survive birth and neonatal period. We monitor closely the newborns and assess tumour development using advanced imaging after weaning. The number of pregnant females subjected to this procedure is less than 100 per year and the total number of embryos, less than 500 per year.

Generation of tumour-bearing adult mice:

In some cases, tumour development will be induced in GEMMs through the activation of genetic alterations postnatally. For example, to induce pituitary tumours in our mouse models of paediatric craniopharyngioma, which is a clinically relevant childhood tumour that is associated with poor quality of life and mortality, we inject tamoxifen into mice to activate the expression of the cancer-promoting genes. This involves the injection of a single dose of tamoxifen. For lung tumours, we deliver a small volume of liquid containing viruses through the nostrils of anaesthetised mice. The liquid travels through the airways into the lung where the virus activate the expression of cancer genes. After the procedure these mice recover very well (in our experience 100% survival).

Sometimes we will need to transplant human tumour cells into immunosuppressed mice (PDX models) to test the effect of novel drugs in tumours comprised of human tumour cells, rather than tumours of murine origin that are generated in GEMMs. For example, we have developed PDXs models of paediatric low-grade glioma, high-grade glioma and craniopharyngioma.

The time for these tumours to develop ranges from 3-4 weeks to 9-12 months. The total number of tumour-bearing mice will be less than 1500 per year.

Advanced imaging. We can visualise and measure tumour development in some of the mouse models we use. We routinely use bioluminescence measurements in our mouse brain tumour models. This involves the injection of a chemical (luciferin) in anaesthetised mice and measurements through a piece of equipment (IVIS). Animals recover very well from this procedure (100% survival). MRI (Magnetic resonance imaging) is also available to us, and we have previously used MRI in preclinical trials in the mice (e.g., when testing new potential anti-cancer drugs). Mice are anaesthetised between 10-20 minutes. We usually need to repeat these measurements every 1-2 weeks to assess tumour growth or regression, in those mice treated with anti-cancer drugs.

The use of advanced imaging is very advantageous. Not only does it help reduce the number of mice in the experiments, but we can also establish closer monitoring in those mice in which we observe faster tumour growth, allowing us to prevent sudden deaths and unnecessary suffering. The number of mice going through MRI or IVIS measurements is less than 1000 per year.

Radiotherapy (RT). Children with high-grade glioma receive RT as the only existing treatment. Usually, the total RT dose is delivered in fractions of smaller doses (referred to as 'fractionated RT') to reduce side effects in the patient. However, RT is palliative and between 3-6 months post-RT, the tumour relapses and the patient dies. As we aim to understand the effects of RT, and the reasons why the tumours relapse, we need to treat the tumour-bearing mice with RT. We use an animal irradiator that delivers radiation, usually in small fractions, locally to the developing tumour in the brain, hence sparing other organs in the mice and avoiding unnecessary side effects. This procedure is done under general anaesthesia and it takes between 10-20 minutes per mouse. Mice recover very well. The number of mice subjected to this procedure is less than 1000 per year.

Injection of substances. Typically, animals will experience mild, transient pain and no lasting harm from administration of substances by injection using standard routes (intravenous, subcutaneous, intraperitoneal). Where administration is required for prolonged periods, animals will be surgically implanted with slow-release devices such as

a mini-pump. These animals will experience some discomfort after surgery and some mild to moderate pain which will be treated with analgesics. The number of mice subjected to this procedure is less than 1500 per year.

What are the expected impacts and/or adverse effects for the animals during your project?

Most of our transgenic manipulations are not expected to cause discomfort, pain or distress to the mice (e.g., breeding) while the genetic alterations are inactive (e.g., in breeders). Once the expression of oncogenic genes is activated, mice may develop tumours, which in our experience are well tolerated and cause no symptoms for several weeks or months.

For example, in the mouse models of paediatric craniopharyngioma, symptoms usually develop after a long period (around 17-35 weeks). Likewise, lung tumours in the mouse do not cause any symptoms for around 4 months and most of our analyses are done around 2 months. Brain tumours are more variable depending on whether they are low-grade or high-grade. Usually, low-grade glioma brain tumours do not develop symptoms in the first 2-3 months, whilst high-grade brain tumours develop symptoms between 3-4 weeks to 9-12 months, depending on the model. Therefore, in general all these tumours are well tolerated and mice do not show symptoms during early and mid-stages of tumour development (weeks or months), which are the stages that we are most interested in studying in this project.

In all cases above, we monitor the mice regularly and as required by the NVS (Named Veterinary Surgeon) and NACWO (Named Animal Care & Welfare Officer). Monitoring includes visual inspection for signs of pain or abnormal behaviour as well as weighing the mice and assessing specific adverse effects (e.g., immotility, head tilting, difficult breathing). Our aim is to avoid the development of adverse effects by regular monitoring and minimise the time the mice experience these effects.

If or when adverse effects have occurred (e.g., neurological signs for brain pituitary tumours and breathing defects in lung tumours or weight loss of 20%), mice are humanely killed. A Table with humane endpoints is included in this licence. Mice showing adverse effects are usually humanely killed as soon as possible and within 24 hours from onset. Mice likely to develop tumours and show signs of ill-being are labelled appropriately and the animal care personnel in our BSU (Biological Service Unit) informed of the expected adverse effects. Phone number of the researchers responsible for the project and other members, including the licence holder is shared with the BSU staff, to act swiftly if the occasion requires to do so.

Expected severity categories and the 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: A proportion of the breeders and GEMMs will not need ear biopsy, hence these will not experience any pain, distress or discomfort. Around 10% of mice.

Mild: The majority of GEMMs will be receive and ear punch for genotyping purpose, and will not experience any further pain, distress or discomfort. Around 60% of mice.

Moderate: Tumour-bearing mice may experience moderate symptoms. Approximately 30% of 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?

Through molecular and cellular studies, we will identify novel drugs to kill senescent and cancer cells, which we aim to test in mice in order to be able to develop and initiate clinical trials in humans.

To develop clinical trials, oncologists, pharma companies and regulatory bodies need to assess that there is a strong rationale supporting their development. Moreover, sufficient preclinical data, including mouse experiments, must be provided to propel preclinical research into a clinical trial in the patients. Therefore, we need to provide evidence of efficacy in experimental animals (mice in this project) before basic research findings can be translated into humans. The mouse experiments that we propose to carry out in this project aim to provide sufficient evidence to develop human studies.

In this project, we will be using mice as experimental model because of the similarities between human and mouse cancers and the availability of excellent murine cancer models. We already have several mouse models of brain, pituitary and lung cancer in our mouse facility. Additionally, we can generate murine models carrying human tumour cells in immunosuppressed mice. Together, our models allow us to study tumours of murine origin and also tumours of human origin. In combination, the in vitro data (e.g., cellular and molecular data) and the preclinical mouse models are very powerful approaches to generate robust data to support the development of human clinical trials.

Which non-animal alternatives did you consider for use in this project?

Although experiments in mice are necessary for the proposed research, other possible alternatives have been and will be explored, including:

Tissue/cell culture: In the last few years, we have developed methods to test the efficacy of many drugs in reducing tumour cell growth in cell culture (culturing cells on a Petri dish). We routinely use cancer cell lines of some of the tumours we study (e.g., paediatric high-grade and low-grade glioma). For other tumours, e.g., craniopharyngioma, there are not any cell lines available, but we have developed and established a new method to maintain small pieces of mouse and human craniopharyngioma tumours in culture on a Petri dish for up to 3 days (tumour explant cultures). This short period still allows us to assess the effects of specific drugs. This approach has had a direct impact in our research and in the numbers of mice used for subsequent studies, because we test in mice only those

inhibitors showing the highest efficacy/lower toxicity in vitro. We will use tissue culture and tumour explants, where analysis of whole embryos or mice is not essential.

Organoids: The ability to grow cancer cells in 3-dimensional structures, which are more akin to the way cells form cancers in mice and humans has provided another platform to study cancer and to assess drug efficacy of potential novel treatments. We are currently working on organoid cultures of high- grade glioma cells and craniopharyngioma. If successful, these platforms will be used to refine and reduce mouse numbers. We are very keen on developing these alternatives approaches in my lab.

Mathematical modelling: In this project, we aim to understand the role of senescent cells in tumourigenesis, and how these cells are able to initiate tumours and make them grow. This study requires the integration of multiple interactions between different cell types (e.g., brain cells, cancer cells, immune cells) and cell states (e.g., senescent cells and dividing cells). To optimise these studies, we will use mathematical modelling, i.e., the use of mathematical algorithms to understand complex cell interactions. The outcome of this approach will be in lined with the 3Rs, as we will be able to refine the mouse experiments and reduce mouse numbers.

Why were they not suitable?

Although in vitro (Petri dish) studies can be used to test how cells become cancerous following gene mutations, this system cannot recapitulate the complex microenvironments existing in developing tissues such as the brain, the pituitary and the lung. Because our approach requires the use of specific cancer-susceptible cell types at specific points in brain and pituitary development, this is currently only possible by using live animals that mirror the complexities and cell populations present in human development.

No cell culture or mathematical model can recapitulate the complexity of a tumour developing within mammalian organs. For example, organoids and cell cultures miss many other cell types that are present in the tumours, e.g., blood vessels, normal brain cells, immune cells or any other supportive cells. In addition, drug delivery to the tumour cannot be modelled in cell culture. In brain tumours, it is important to consider the potential problem of the brain-blood-barrier (BBB), which can be tested only in live mice. Because fruit flies and nematode worms lack immune cells, a functional blood brain barrier, the full complement of brain and lung cellular diversity, and the whole-body context of a mammal, they cannot generate the data most crucial for our work.

Tissue and cell culture models (including organoids) are suitable to screen drugs, determine efficacy of new drugs and understand molecular mechanisms of efficacy. Mathematical modelling can also help to make hundreds of computer experiments to recognise specific patterns in the system and optimise animal experiments. However, at the end of this discovery path, new treatments need to be tested in experimental animals, in this PPL in murine tumour models, to assess their efficacy and potential toxicity. Regulatory and research bodies require preclinical assessment of potential therapies in animal models prior to their translation into the clinic.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise

numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

I have used our annual return of procedures data to estimate the number of animals that we will be required in the next 5 years. As I am planning to continue working on similar projects, I estimate that number of tumour-bearing mice, breeders, etc will be similar to the previous 5 years.

We have maximised our breeding strategies to generate the right experimental mice with minimum waste of mice with unwanted genetic alterations. Efficient colony management ensures that only mice that are actively being used are mated and produce offspring. Those mouse lines that are no longer required are cryopreserved and closed at the earliest opportunity. The licence holder routinely carries out 'mouse audits', which involve visits to the animal facility to check the status of the mouse stocks.

When we started doing preclinical experiments to test the potential efficacy of new chemicals more than a decade ago, we consulted a statistician. Now, we have acquired a considerable expertise in designing and conducting these experiments, as well as in data analysis. When dealing with a new chemical that we have not used before, the first step is to perform a literature search to identify whether the research that we are planning to do has already been done in mice. If not done, we use the relevant literature to guide the experiment; for example, we usually obtain information regarding the maximum tolerable dose, administration routes, drug preparation and stability, maximum concentrations in the blood, potential adverse effects in the mice, etc. Often, we also contact other researchers in the field that have used the particular drug or even the drug supplier, to gather this essential information. Then, we will design a pilot study, usually with just 2-3 mice per group to test a couple of doses of the drugs and to assess toxicity and efficacy.

After the pilot, we design a larger experiment that will be statistical powered to test a clear hypothesis; usually whether a particular drug can reduce tumour burden or kill senescent cells. Based on our experience, acquired in our previous licence, we know that usually 8-10 mice per group is sufficient to get statistical power to test the hypothesis. We use suitable statistical methods to ensure that the work will yield reproducible and statistically sound information, for example when determining the group size. Mice are usually randomised when the experiment is designed, but we use males and females in the experiments and the groups are balanced for sex and age. We always include a control group that will be treated in exactly the same manner, but will not receive the drug (only the vehicle where the drug is dissolved). Although experiments are usually not blinded, the data obtained by the researcher conducting the experiment is thoroughly discussed and scrutinised in lab meetings. Data may include tumour volume, number tumour lesions, number of tumour and senescent cells, survival, etc. Data is analysed using suitable statistical methods.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

To reduce the number of breeders, we discuss in our lab meetings the genetic crosses to generate the experimental mice but minimising waste of mice. For example, establishing a breeding strategy that generates only or mostly relevant genetically altered mice for our research.

For testing new drugs in tumour-bearing mice, we employ basic statistical methods to calculate the sample size for these experiments. Usually, we do not know the variability in the response to a drug, therefore we tend to use a 'best and worst-case scenario' to assess a range of number of mice that will provide sufficient statistical power (in our experience, this is usually 8-10 mice per group).

We also ensure that the drug to be tested is the one that we will subsequently seek support to develop a clinical trial, therefore reducing the need to duplicate experiments in mice. The selected drugs are discussed with clinical oncologists to ensure that we use clinically-approved drugs, therefore facilitating translation into the clinic.

All preclinical studies are thoroughly planned and discussed in our lab meetings to ensure that robust data are obtained. Hypotheses are clearly articulated and the analysis at the end of the experiment is planned to maximise the amount of data obtained per animal. For example, we will combine objective quantitative analyses carried out by a machine with histological analysis performed by the researcher. The idea is to ensure that the hypothesis is tested thoroughly and without bias. For example, for the last few years we have refined these experiments by developing methods to visualise and quantify tumour growth in a living mouse. This has contributed to reducing the number of mice we use in the experiments. Any excess of tissue or cells is frozen to be able to re-analyse the samples if required, without repeating the experiment with mice.

We also discuss humane endpoints and establish early endpoints for the experiments. For example, if testing a drug for its capacity to kill senescent cells, we humanely cull the mice before appearance of any symptoms. If we aim to assess whether a particular drug can reduce tumour growth and extend mouse survival, we will not use death as the endpoint. On the contrary, we will set up early humane endpoints that consider a number of ill-health indicators (e.g., behaviour, appearance, response to stimulus, weight loss (or body conditioning) and specific clinical signs (e.g., breathing difficulty in mice developing lung tumours or tremors in mice bearing brain tumours)). We conduct our studies considering the ARRIVE ((Animal Research: Reporting of In Vivo Experiments) 2.0 guidelines.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

As I have previously mentioned, we have optimised our breeding strategies to avoid waste of mice. We carry out pilot studies when testing a drug for the first time. We are also planning to do mathematical modelling in a new project (see sections above). Our animal facility is currently establishing an ' Animal Tissue Exchange', which we will use to donate mice and tissues at the end of 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.

Genetically altered mice offer the most incisive approach to the analysis of birth defects and cancer mechanisms because:

Mouse genetics is understood almost as well as in humans, offering the best possible means for genetic analysis in a mammal.

Birth defects and cancer in genetically-predisposed mice closely resemble those in humans, providing excellent models for analysis.

Transgenic/gene knockout technologies offer a sophisticated route towards studying the effects of genes in particular tissues, or at specific stages.

We will breed and maintain mouse models of paediatric low- and high- grade glioma, craniopharyngioma and lung adenocarcinoma (adenocarcinoma is a type of malignant cancer). All of these mouse models have previously been characterised, validated to be relevant to the human cancer and published. For example, the genomic alterations they carry are the same or functionally equivalent to those observed in the human tumours. We will also use mouse models we have previously generated, validated and published. Finally, we will use immunosuppressed animals to grow human tumours in the mice, by transplanting human cancer cells into the brain of the mice. These mice need to be immunosuppressed to avoid rejection.

Most of our transgenic manipulations are not expected to cause discomfort, pain or distress to the mice. Once the expression of oncogenic hits is activated (usually by administration of a chemical), mice will develop tumours, which in our experience are well tolerated and cause no symptoms for several months. For example, craniopharyngioma is usually asymptomatic for over 17-35 weeks, lung tumours for around 4 months and brain tumours for over 6-9 months for benign tumours (according to our collaborators and published literature). Even malignant brain tumours are well tolerated and mice do not show symptoms for weeks/months. During this time, tumour development is assessed and mice are monitored regularly (i.e., mice are checked daily and their general aspect, behaviour, weight, responsiveness to stumuli, etc, are assessed by trained staff).

We have refined many of the methods during the last 10 years and will continue to do so. For example, we have optimised the protocols we use to induce tumours in the mice to reduce adverse effects and avoid premature death due to excessive tumour burden. We have also established methods that allow to determine tumour size and the effect of the tested drug in reducing tumour size without killing the animals, e.g., using MRI (magnetic resonance imaging) or bioluminescence imaging (BLI). BLI is based on the production of light by tumour cells; the more light is generated the larger the tumours.

Patient-derived xenograft models, which develop human tumours, and genetically-engineered mouse models (GEMMs) of brain, pituitary and lung human tumours are generated through manipulations under general anaesthesia (as appropriate for the technique) and analgesia. This is done in designated areas using aseptic technique to prevent infections or any complications. In cases where mouse survival is evaluated, for example when assessing the anti-cancer efficacy of a particular drug, early humane endpoints are established. We do not use mouse death and an endpoint.

If unexpected adverse effects should develop (e.g., neurological signs for brain pituitary tumours and breathing defects in lung tumours), the mice are humanely killed. Mice likely

to develop tumours and showing signs of ill-being are labelled appropriately and the animal care personnel in our BSU (Biological Service Unit) informed of the expected adverse effects. Phone number of the researchers responsible for the project and other members, including the licence holder is shared with the animal care personnel in our BSU (Biological Service Unit) to act swiftly if the occasion requires to do so.

Why can’t you use animals that are less sentient?

Less sentient animals such as zebrafish, fruit flies and nematode worms cannot recapitulate the uniquely mammalian biology of the human organs. This is especially relevant to the development of new treatments for brain tumours, which have to be analysed for their effects on not only the tumour, but also on normal brain function and other critical organs in the body. These treatments also have to gain access to the brain through the blood brain barrier. These "extra challenges" facing brain and lung cancer research cannot be studied in less sentient animals because they either do not occur in them or are much more challenging to address. However, they do occur in mice and are easily observed in mice. Therefore, mice are the most appropriate, least sentient animal model for studying and developing new treatments human tumours.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Mice are kept in a facility that is fully dedicated to maintain mice for research. Mice are usually grouped (except males that have been used as stud males, as they will fight if put back together with other males) in individually ventilated cages. They have permanent access to food and water, and are kept are at optimum temperature and humidity. Their environment is enriched with bedding, nesting material, refuges, etc.

Xenograft mouse models or GEMMs with somatic mutations in the brain, pituitary and lungs are generated through manipulations under general anaesthesia (as appropriate for the technique) and analgesia. This is done in designated areas using aseptic technique to prevent infections or any complications.

Radiation is normally delivered in small doses during 1-2 weeks, rather than a higher single dose. This is better tolerated by the mice and it is more akin to the radiotherapy regimen used in patients with cancer. It also reduces the amount of radiation that is delivered to other organs, hence reducing further side effects. The use focal versus total body radiation has been an important refinement in the previous licence.

Induction of tumours in susceptible mice has also been refined. For example, by administering lower number of virus in the case of murine lung tumours or lower concentration of the activating chemical for brain and pituitary tumours. This refined procedure still allows us to address our research questions but minimises the risk of having a too high tumour burden in the mice.

We have also established protocols to be able to follow tumour development using advanced imaging, for example MRI and BLI measurements. This means we get quantitative data on tumour growth over time in the individual mice, with a beneficial effect in both reducing number of mice used in the experiments and improving the quality of the data obtained.

What published best practice guidance will you follow to ensure experiments are

conducted in the most refined way?

Work in this project will be undertaken in accordance with the principles set out in the Guidelines for the welfare and use of animals in cancer research: British Journal of Cancer (2010) 102, 1555-1577.

LASA guidance for the application of aseptic techniques during surgery. We also follow the ARRIVE 2.0 guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Through constant interaction and engagement with veterinary and animal husbandry staff at the facility, attendance to the annual NC3Rs meeting and through receipt of electronic and printed media from NC3Rs on ongoing improvements to techniques and methodologies. If needed, amendments to the licence will be applied for, if new, more refined procedures are needed to improve animal welfare.

81. Therapeutic Antibody Discovery

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, Therapeutic, Immunisation, Monoclonal

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We will generate new antibodies to become the medicines to treat diseases where current treatment can be improved, and also for the new disease treatments as they are identified.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 many diseases where the current treatment can either be improved (e.g., cancer) or where existing treatments are becoming ineffective (e.g., bacterial diseases resistant to multiple antibiotics.). With our capabilities, we can in many cases produce monoclonal antibodies that can either become a new treatment for a disease; replace an existing medicine because it is better for the patient treatment; or an addition to the current treatment that is used in combination to improve the lives of patients.

There will always be the need to find better medicines for existing diseases that change over time (e.g., microbial diseases, cancer, asthma, etc) and new medicines for emerging diseases (e.g., Covid)

What outputs do you think you will see at the end of this project?

New antibodies will be made to the support the discovery and development of new medicines. Increased knowledge of immunisation protocols for generating antibodies.

Dissemination of knowledge in this field to other scientists through presentations and/or publications.

The aim will be for antibodies to be developed into medicines to treat patients for diseases where there is either no treatment or where the current treatment can be improved.

This licence will be used to generate new medicines to disease targets. A panel of in-vitro and in-vivo scientists will review the target and desired antibody to determine if the work is done by in-vitro or in- vivo methods. This will include a literature search on pre-existing antibodies to the target and the methods by which they were made.

Where non-animal alternatives are not viable, we will immunise mice to produce an immune response and screen immune tissues (spleen, lymph nodes, bone marrow and blood) to identify any antibodies that could be potential new medicines. An internal scientific review forum will review the final immunisation plan and endorse it prior to any work commencing.

The generated antibodies are tested in multiple ways to identify the best one to be used as a medicine to give to patients. Success of our group is measured by how many antibodies we discover that have the potential to be used as medicines in patients. Our aim is to be successful in this regard for all our projects.

As part of making new medicines, we are also improving the methods we use to do this. We constantly evaluate ways to make the immune response better by producing more useful antibodies, faster, with fewer immunisations and milder immunisation methods. Success in this aspect would be identifying better methods that can be used in future immunisation projects to reduce animal numbers, reduce immunisation duration, and provide better animal welfare. Many of the disease targets in our scope are difficult to work with and have challenging demands of the antibody to make it usable in patients.

Where these improvements do not compromise our intellectual property and contribute towards the 3Rs or scientific advancement we will look to identify opportunities to share this knowledge with the scientific community through publications, conference presentations or university collaborations.

Who or what will benefit from these outputs, and how?

Patients ultimately benefit from this work. Our expertise and commitment has ensured that medicines to treat patients have been discovered and developed at the company.

Patients who receive these medicines can have life changing outcomes as a result of our work. Obviously with diseases like cancer or microbial diseases (bacterial infections, viruses) the patients may be completely cured or have their life extended by a new

antibody medicine. But there are also debilitating diseases (e.g., asthma, psoriasis, dementia, Alzheimer's and many others) where new antibody medicines can enable people to have a greatly improved quality of life enabling them to lead a more normal (if not completely normal) life.

Any immunisation refinements from previous licence work or as part of this licence will be applied to new immunisation projects.

The scientific community will benefit from the release, and thereby increase, of knowledge through publications or conference presentations or university collaborations.

How will you look to maximise the outputs of this work?

As part of our work, we do attend internal and external meetings and conferences. Presenting posters and giving presentations on our work, (especially in the areas of challenging targets and demanding antibody requirements), is something that we aim to do. This not only enables us to keep abreast of the latest developments but also to share some of our knowledge.

I have presented data at international conferences on data that has been generated from work on my previous antibody discovery licence.

The company also conducts PhD collaborations with universities to enable the training of future scientists as well as investigate our immune responses from a more fundamental perspective. We utilise the ability of PhD students to do investigations that otherwise would not be done within the company that can further our understanding of immunology.

Where immunisation improvements we make do not compromise our intellectual property, and contribute to 3Rs or scientific advancement. we always lookto identify opportunities to share this knowledge with the scientific community through publications or conference presentations or university collaborations.

Our introduction of slow-release formulations has improved immune responses and increased the numbers of immune cells. This greatly improved the success rate of identifying potential new medicines.

Species and numbers of animals expected to be used

- Mice: 900

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are using mice as they have a very similar immune system to human beings enabling us to reliably predict immunisation outcomes to our disease targets.

The mice we use have been genetically modified to produced 'human' instead of 'mouse' antibodies, so that these can be used straight away for medicine discovery without the need to convert them from 'mouse' to 'human'.

We only use adult mice and they are only immunised only after they have reached a minimum of 8 weeks of age such that they have a mature immune system that will give the best immune response.

Typically, what will be done to an animal used in your project?

Immunisation involves giving a substance to an animal usually by injection into or under the skin while the animal is anesthetised.

In our rapid immunisation (Protocol 1) mice will then be immunised 3-5 times over 7-28 days with at least than 2 days between each immunisation depending on the project requirements. For each immunisation the mice will be usually be anaesthetised and injected under the skin. When it is appropriate animals may be given an immune modulator(s), usually under the skin. This can be prior to, with or post immunisation to boost the immune response. At the end of the immunisation period the mice will be humanely killed followed by removal of immune tissues for screening.

The number of immunisations done in protocol 1 will depend on the duration of the protocol:

Protocol duration (Days)	Maximum number of immunisations
7	3
10	4
14+	5

In our conventional immunisation (Protocol 2) mice will then be immunised, usually under the skin, once every 2 weeks for typically 4 times (but up to a maximum of 6 times). A blood sample may be taken at least 7 days after each immunisation to assess the immune response and determine whether further immunisations are required. When it is appropriate animals may be given an immune modulator(s), usually under the skin. This can be prior to, with or post immunisation to boost the immune response.

When the appropriate immune response has been achieved the mice will be humanely killed followed by removal of immune tissues for screening.

What are the expected impacts and/or adverse effects for the animals during your project?

For both immunisation protocols a small number of animals can exhibit some general signs of being unwell for a short time (up to 2 hours) after immunisation: for example, they may show reduced activity or starey coat. Some mice (50%) may also experience weight loss. These mice will be weighed daily and given supplementary food until their bodyweight recovers (typically within 48 hours).

Rarely swellings, fur loss or scab formation can occur at the site of immunisation. This normally resolves itself in up to 7 days without any intervention being required.

Very rarely, within 1 hour after the animals have been immunised, some animals may suffer an allergic reaction where animals will develop difficulty breathing. When this happens, animals will be continuously monitored and if there is no change in the animal's status after 30 minutes the animal will then be euthanised immediately.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Based on our immunisation methods all animals can experience up to moderate severity.

Mice – 100% - Moderate

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Where we use animals to generate antibodies it is because for one or more reasons the target is incompatible with the in-vitro antibody discovery methods. This can be due to the nature of the target (e.g., membrane targets), the reagents that can be generated and their compatibility with the in-vitro platform being used.

Which non-animal alternatives did you consider for use in this project?

Our company operates state of the art in-vivo and in-vitro antibody discovery platforms. These are considered on a 'target-by-target' basis for compatibility and probability of success. We routinely use yeast display methods as the comparator for in-vitro antibody discovery.

Every request for new antibodies is assessed to ensure that all possible alternatives that do not involve animal use have been considered before the use of animals is allowed. Based on the project requirements, historical knowledge, target type, reagents available and platform capabilities a decision is made as to which platform will be used.

This assessment, called the 'Platform Decision Meeting', considers all the requirements of the antibody discovery campaign and which platform is best to obtain the required antibodies. The meeting is attended by antibody discovery experts in in-vitro and in-vivo methods, protein expression experts to provide input on what antigens and/or screening reagents can be made, and antibody assay experts to provide input on how antibodies of therapeutic relevance will be identified.

- **Does an antibody for this application already exist?**

- If so, can this be licensed for the project Antibody requirements:
- **Can the in-vitro methods isolate the desired antibody?**
- **Can in-vivo methods generate the desired antibody?**
- Discovery reagents
- **Are the reagents suitable for in-vitro methods?**
- **Are the reagents suitable for in-vivo methods?**
- Precedence of antibody discovery

Have antibodies been made to this target before and how was this done?

Is there evidence that supports in-vitro or in-vivo antibody discovery?

The results of this assessment are used to ensure that animals are used only if required. The outcomes of this meeting are documented and stored for retrospective assessment as required.

Why were they not suitable?

We are committed to only use animals when a non-animal method cannot, or is very unlikely to, deliver the required antibodies.

Our targets are identified by our disease research scientists, and we therefore do not know what they will be at the point of applying for this licence.

Therefore, on a 'target by target' basis the requirements of the project are compared to the reagents available and the capabilities of the in-vitro and in-vivo platforms. Based on this analysis the best platform is chosen to be used.
In very rare cases there may targets where no platform that has a high probability of success for a target by lack of precedence in the scientific community, prior experience with the same target class or limitations of the reagents available. In these very rare instances both platforms may be used in parallel to maximise the chance of obtaining a therapeutic antibody in the minimum timeframe to develop the medicine for the patients.

Principally the reagents available for the target for immunisation and/or selection can define if in-vitro or in-vivo is the best way forward. If the reagents are not able to be produced in a format that is compatible with the in-vitro method chosen (available) then in-vivo may be used.

For example, sometimes it was not possible to produce a form of the target that is compatible with in- vitro methods. This has been due to proteins not expressing, being sticky, or not having activity. When this occurs, we cannot deliver an antibody with the desired properties using in-vitro methods and in- vivo methods are the alternative way of generating the antibodies required.

The in-vivo immunisation process can also utilise genetic immunisation methods, cell immunisation methods and direct screening on cells to overcome all the above problems.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

This is based on the current frequency of therapeutic antibody discovery projects and the average number of animals required per project.

Based on our previous work and extensive experience, we typically have 4 (minimum 3 and up to 5 in exceptional cases) animal immunisation groups per target. Each immunisation group is different in the strategy and/or reagents used. Each of the immunisation groups has either 4 (protocol 1) or 7 (protocol 2) mice per group. This ensures we have enough immune tissues for our downstream work to identify the small percentage (0.1-1.5%) of antibodies from the total immune response that have the potential to be a medicine for patients.

Where we are also testing an immunisation parameter, one mouse group is the baseline group to compare and assess the outcome. This enables us to know if changing the parameter delivered an improvement. Mice are randomized into each group using a using an in-house random number generator function to ensure there is no unconscious bias.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

To minimise the number of animals not used from our transgenic mouse colony, we use males and female in our immunisation projects. Typically, in the literature, immunisation work is described as using only female mice and within a defined age range.

The consequences of this are:

Male mice are not used, and more breeding is required to generate the required number of female mice for each immunisation campaign

Mice are not used if they exceed a defined age range.

We have shown that both sexes of mice and that a wider age range can be used in antibody generation without compromising the quality of the immune responses, and thus greatly reducing the number of animals bred for our work.

As targets are identified by our disease research scientists, we therefore do not know what they will be upfront. Therefore, when a project has passed the criteria in our ‘Platform Decision Meeting’ that initiates an in-vivo antibody discovery project, the target and reagents for immunisation and screening are examined.

Historical knowledge of immunisation outcomes and success rates for previous targets of the same ‘target class’ can also influence the immunisation strategy, e.g., for immunisation strategies that have been previously successful for a related target, may be able to be

applied to the current target. This has been done with chemokine receptors and ion channel target classes.

Searches of the scientific literature or pre-existing patents relating to the specific target can also give information that can help define immunisation strategy. All of this knowledge is combined with our own expertise to develop the final strategy. Simply copying what has been done previously or by others is not a default choice as:

We may be looking for a different antibody or a better one that what is described in the patents/literature.

Our methods may use less animals and/or have better animal welfare

We may have access to new/different reagents that allow for better immunisation options.

'Robust Study Design' guidelines and 'Design of Experiment' principles are used in the design of the immunisation plan.

Once complete the immunisation plan, number of animal groups and number of animals per group are peer reviewed in an open scientific forum meeting with other animal users, vets, statisticians, and other scientists able to view and comment. This enables a robust and open discussion to ensure that the desired antibodies can be delivered, the number of animals proposed is justified and proportionate. If required, changes are made to the immunisation plan and it is then represented to the review forum until it is finalised.

The combination of our extensive antibody generation experience, immunisation refinements in our immunisation techniques and peer review results in the minimum number of animals that are needed are used to generate antibodies to make medicines to treat patients.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We collect all animal data on our immunisation procedures to help us to identify what makes immunisation more successful. Our data analysis tools (Spotfire, Excel, etc) allows us to create custom dashboards to understand outcomes further and detect any trends that lead to successful outcomes.

This knowledge has then been applied to reduce the numbers of animals immunised for new projects where possible.

We have a standardised animal group size for each immunogen used, and a maximum number of animal groups per target to ensure that animal number used per therapeutic target are controlled.

Once the desired immunisation response has been achieved, we harvest all types of immune tissue to maximise our chances of finding the right antibodies.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the

procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We use immunisation methods to generate immune responses to our disease targets. Mice will be immunised using methods similar to human vaccination :- injection of substances usually under the skin, but sometimes into the peritoneal cavity or by intravenous injection.

Our methods and reagents that we use are chosen with intention to have minimum impact on animal welfare during the immunisation period.

If during a study, adverse effects due to a specific reagent/technique are observed, discussion with subject matter experts on what options there are to either remove the reagent or change how it is used are held.

Where we judge the impact on animal welfare of using specific techniques or reagents is too great, these will be discontinued from the current and any future immunisations. This has occurred during the previous licence after it was shown that careful monitoring with provision of supportive therapy, e.g., warmth or supplementary feeding if required, was not sufficient.

Non aversive handling methods are used when immunizing animals. We also have a custom designed restrainer for mice to minimise the effect of handling the mice.

Animals are briefly anaesthetised for restraint when immunised to minimise distress due to the number of injections required.

Environmental enrichment is provided for all our animals in their cages.

Why can’t you use animals that are less sentient?

It is necessary for the immune system in the animals to be working normally, and to ensure this, that the animal has achieved adult stage. Immunisation and the immune response are a process that can take weeks/months to mature and as such it is not possible to do with immature, less sentient or terminally anaesthetised animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

As part of our work, we are always looking for refinements that improve the animal welfare without compromising the quality of the immune response.

Our animals are closely monitored after immunisation at 5 specific timepoints over 4 hours in case to check on their condition. If required, there is a heat mat to help recovery and softened food is provided. If required the time between each immunisation can be adjusted to give more recovery time.

In the previous licence the refinements were:

- New techniques for genetic immunisation using mRNA methods with lipoparticles that are more refined than previous methods (e.g., genegun and hydrodynamic immunisation) and have also enhanced the quality of antibodies generated. This has had a major impact in identifying novel antibodies in our projects.

- Investigating new adjuvants as part of our aim to elicit the best immune response without side effects and minimal impact on animal welfare. I have introduced the use of agonists of the “stimulator of interferon genes” (STING) protein. This has been shown to still give good immune responses whilst improving the animal welfare by not having the side effects or other adjuvants. As a result of this we have stopped using adjuvant combinations that, whilst giving good immune responses, can cause more adverse effects.

- Investigating and introducing slow-release antigen formulations. These have been shown to improve the immune response and give rise to increased quality antibody responses in a shorter time period.

These refinements have made it possible to prosecute targets that would have otherwise been intractable by in-vitro and in-vivo antibody discovery methods.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

As stated, within the company we have updates and dissemination of all 3Rs advances and best practice in the in-vivo field. We have implemented changes to diet, husbandry and methods of monitoring to ensure that are animals have an optimised standard of care.

Our work uses Robust Study Design principles for designing the immunisation strategies, e.g. randomisation, blinding and advice from biostatisticians on study designs.

We work to the principles of PREPARE guidelines (PREPARE: guidelines for planning animal research and testing - Adrian J Smith, R Eddie Clutton, Elliot Lilley, Kristine E Aa Hansen, Trond Brattelid, 2018 (sagepub.com).

Our techniques for immunisation also work to recommended practices from other experts in the field - Diehl 2001 (A good practice guide to the administration of substances and removal of blood, including routes and volumes - https://analyticalsciencejournals.onlinelibrary.wiley.com/doi/10.1002/jat.727).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Within the company we have regular updates and dissemination of all 3Rs advances in the in-vitro and in-vivo fields of antibody discovery. There is a hub specifically for 3Rs information including links to external sites (NC3Rs, Norecopa) for information and updates to alternatives and other 3Rs information.

Attending conferences also helps me to keep up to date on in-vitro antibody discovery, immunisation technique refinements and improvements.

Within my department working alongside in-vitro antibody discovery scientists I am intimately involved in the triage of all disease targets that come for antibody discovery for selection of the antibody discovery platform (in-vitro vs. in-vivo).

Routine review of the literature (Google searches, PubMed, Patents) for new information by searching for “in-vivo immunisation”, “mouse immunisation”, “mouse immunisation welfare” and related search terms. Attendance at scientific conferences to keep up to date in the latest antibody discovery processes (in-vitro and in-vivo).

Combined these activities ensure that I am aware of 3Rs advances that can be applied to this project licence. My contacts internally have led to the use of more refined genetic immunisation methods and adjuvants that reduce the impact of immunisation on animal welfare.

82. Toxicity of Non-Pharmaceuticals in the Rabbit

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

Rabbit, Toxicology, Irritancy, Non-pharmaceuticals

Animal types	Life stages
Rabbits	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to determine scientific and/or regulatory endpoints in rabbit toxicity, including:

immunotoxicity,toxicokinetics/toxicodynamics/biodistribution/persistence/ biomarker and supplementary/investigative toxicity, tolerance and/or safety.

The test materials under investigation will be non-pharmaceuticals (e.g. agrochemicals (including microbial pest control agents), food additives/foodstuffs and industrial chemicals, potentially including household product ingredients.

No cosmetic products or chemicals that are exclusively intended to be used as ingredients in cosmetics will be tested.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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.

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 rodent, non-rodent and non-animal studies in demonstrating to governments and the public the safety of these substances.

What outputs do you think you will see at the end of this project?

The overall benefit of this project is that it supports the development of safer more effective agrochemicals or food additives/foodstuffs, safer usage and transporation of industrial chemicals and biocides and the economic benefits from production/use of these substances, by generating high quality data that are acceptable to regulatory authorities and enables internal decision making within our clients' organisations.

Achievement of the objectives of this licence will enable safer agrochemicals, food additives/foodstuffs, industrial chemicals and biocides to be approved for marketing/registration and for the authorities to prescribe conditions for safe use of these substances and will also help to remove unsuitable candidates from the development pipeline at an early stage, thus saving animals and resources._

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 products under development and, where appropriate, to satisfy governmental regulatory requirements necessary to gain registration or marketing authorisation.

Humans and animals will benefit from these studies as this work will contribute to the development of safer or more effective agrochemicals or biocides, safer food additives/foodstuffs, safer usage of industrial chemicals and the economic benefits from production/use of these new substances, or identification of substances that are deemed unsafe for further development. We may, by our work, also contribute to better knowledge and understanding of these types of chemicals, and that knowledge may be used to develop further new chemicals.

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 regulatory purposes or to support regulatory purposes (e.g. to support marketing approval or registration). Previously however, we have collaborated with customers and shared data we have produced in the form of scientific publications that are in the public domain.

Species and numbers of animals expected to be used

- Rabbits: 17000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Regulatory authorities require the initial use of rodents and a second, non-rodent, species; the rabbit is often employed as the non-rodent species of choice. The rabbit behaves similarly to humans following exposure to irritant substances and as such they are used on studies that look for irritant effects of a test item.

Adult animals are used throughout this project.

Typically, what will be done to an animal used in your project?

Substances will be administered to the animals by standard routes of administration such as oral (gavage, and treated diet or drinking water) or direct application to the skin or eye.. For non- pharmaceuticals, the route of administration will usually be by the potential route of human exposure (e.g. oral, dermal, inhalation).

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 done by a doctor.

Animals may be restrained (either held by the technicians or by wearing bandages, jackets, Elizabethan collars or stocks) to aid administration of substances or to prevent the animal interfering with the administration site.

At the end of the study, the animals are normally humanely killed and subjected to post mortem examination with tissue samples being taken for analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

Whilst on study, the animals may experience reactions to treatment such as decreased activity, changes in posture or gait, decreased appetite, weight loss and breathing irregularities. Whilst death is not the aim of any of our studies the test substances used may have unknown effect on animals. We endeavour to avoid severe effect due to toxicity by using small test groups initially to aid in setting dose levels for main studies and if no data are otherwise available for the particular test compound we may extrapolate from other species where the same compounds has been used or from other rabbit studies using similar compounds if such data are available. For all studies we closely monitor animals and if predetermined humane endpoints are reached based on signs observed we humanely kill to avoid unnecessary suffering.

Experience shows that around 80% of animals show subtle or mild signs (such as subdued behaviour); moderate signs (such as 15% weight loss) may be seen in around 20% of animals, usually in the high dose groups. A very few animals (<1%) may show severe signs (such as fitting). Most dosing techniques, manipulations or investigations do not

cause lasting adverse effects, but a small number of animals may show transient mild or moderate distress (such as withdrawal of blood or administration of a mydriatic).

Expected severity categories and the 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 numbers from the last project around 80% of animals would be expected to show mild clinical signs, and 20% of animals moderate signs (high dose groups or had a surgical procedure).

It is impossible to predict the proportion of severities expected on a service licence like this, as this will be dependent on what study types we are asked to perform, however, a distribution between ‘mild’ and ‘moderate’ severities similar to those in the last project are anticipated.

Although there are severe severity procedures on this project, on the last project less than 1% of animals experienced severe clinical signs.

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?

Although non-animal (in a test tube (in vitro) or computer modelling (in silico)) studies can provide useful supporting data to refine and reduce animal studies, definitive assessments of whole body or 'systemic' exposure, efficacy and toxicity can only be achieved in studies using intact animals.
This is because each body system does not act alone but is part of an overall interrelated biological system with influences from other organs within that system. To date it is not possible to model a whole body system in the laboratory and the use of animals in regulatory toxicology, and this remains a mandatory legal requirement.

We will, however, remain vigilant to seeking alternatives where possible and where such tests are available, use validated non animal alternatives (for example to screen out, using cells in a dish rather than a whole animal, substances that are shown to be corrosive such that work does not further progress into animals.

Currently, however, for many of the study types within this project, there is unfortunately no scientific, ethically or legally accepted non-animal alternative available.

These studies are run to satisfy the regulatory requirements of governments around the world to ensure agrochemicals, biocides, food additives/food stuffs and industrial chemicals are safe for humans and animals. These tests are very specific as to what they require in terms of testing in animals to ensure this.

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 regulators, 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?

There is no other non-animal alternative 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 toxicity but cannot yet replace in vivo (animal) studies.

No study in animal is conducted under this licence until an assessment has been made to determine that the specific study is necessary and justified, i.e. the study aims and objectives are consistent with the scope and purpose of the licence and cannot be achieved by any other means not involving the use of animals. This assessment will involve consideration of any potential non-animal alternatives, review of existing data on the test item and reference to any other relevant information (including literature review, in-house data, information on similar items).

Why were they not suitable?

Although there are in vitro 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 in vitro tests that brings all these complex events together, as in the whole (animal or human) organism.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

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. It is, however, impossible to accurately predict the number of studies that may be performed, in the circumstances.

The numbers of animals used in each study are in some cases specified in the regulatory guidelines; where not specified, numbers are based on established minimum regulatory expectation, or on scientific estimates of the minimum numbers required to meet study objectives.

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.

Before we embark on animal testing we ensure that we have all the relevant data to hand either from early phase studies that either we ourselves have completed (or are available from the sponsor) or from seeking further information in literature. We take a staged approach to testing such that if a compound is shown to be unlikely to be effective or shows unacceptable toxicity via proposed route of administration it can be removed from the testing programme at an early stage and not further progressed thus avoiding 'waste' of animals.

For later phase studies, the numbers of animals used are kept to the minimum that would comply with regulatory requirements.

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 use smaller groups of animals to get an idea of the doses) we need to use for the main studies. These preliminary studies are important as they give us confidence that the doses we are using are correct prior to testing them in bigger groups of animals as required by global regulators. Preliminary studies are often used.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

This project uses adult rabbits

For studies that assess the effect of a test substance to cause irritation before the study starts we will undertake a weight-of-evidence analysis using all available information on the material. Such information will include chemical characteristics of the test substance and, where available, results from non-animal studies and computer modelling. The results of this analysis will be used to offer a prediction of the potential of the test item to cause a severe effect on an animal and the need for a live animal study. When acceptable to the regulatory agencies such studies will be conducted by an appropriate in vitro method.

Sequential testing, with review of findings at each stage and modification of subsequent stages as necessary, maximises opportunities for refinement to achieve the desired scientific endpoints with the least risk of pain, suffering, distress or lasting harm to the animals.

Animals are monitored for clinical signs of toxicity or other effects on their health and wellbeing, and in order to prevent unnecessary suffering, humane end-points are applied under appropriate veterinary guidance (e.g. modification/withdrawal of treatment with the test substance, provision of palliative or therapeutic treatments, or humane killing of affected animals).

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 advice and on the occasions we have to anaesthetise the animals and for general advice on animal welfare.

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 deeply unconscious then we do so.

The rabbit is a naturally social species, living together in groups in the wild. Accordingly where possible, rather than house animals individually, we will (specifically with female animals who are less inclined to fight when housed in single sex groups than males) house female animals in social groups of two or three individuals.

Where more than one method of assessment exists, the least invasive method /most 'refined' method will be employed (for example for the recording of body temperature we

will use a human/paediatric thermometer that can be held on ear of rabbit or subcutaneously implanted microchip responder rather than using a thermometer than is inserted into the rectum (the use of a rectal thermometer requires justification to the PLH, NVS and NACWO)).

Refinements under the previous programme of work PPL have included:

the use of refined methods for the collection of body temperature (thermometer for use on ear) and microchip transponder under skin as the primary methods.

the twice weekly offering of a small quantity of vegetables as a dietary supplement rather than feeding of pelleted rabbit food alone.

the group housing (normally in social groups of two or three) for female rabbits.

the inclusion of shelves in all cages to provide 3D complexity to the cage, enabling an animal to explore the full height of cage or to rest in the area under the shelf if desired.

Why can’t you use animals that are less sentient?

Regulatory authorities require the initial use of rodents and a second, non-rodent, species; the rabbit is often employed as the non-rodent species of choice (in preference sometimes to the dog or pig)
The rabbit behaves similarly to humans following exposure to irritant substances and as such they are used on studies that look for irritant effects of a test item.

Most of these studies require repeat dosing for days, weeks or months, to assess potential adverse effects in man, 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?

Animal welfare is of utmost importance to us.

During dosing and restraint, animals are constantly and closely watched for signs of distress. If equipment is used to enable us to achieve the scientific aims of the study, then we would habituate animals to this equipment prior to dosing. 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 deeply unconscious 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.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Diehl et al (2001). A good practice guide to the administration of substances and removal of blood, including routes and volumes. Journal of Applied Toxicology, 21, 15-23

OECD Test No. 405: Acute Eye Irritation/Corrosion OECD Test No. 404: Acute Dermal Irritation/Corrosion
OECD Guidelines for the testing of chemicals, Section 4: Health Effects OPPTS Health Effects Test Guidelines
OECD ENV/JM/Mono (2007). Guidance document on the recognition, assessment and use of clinical signs as humane endpoints for experimental animals used in safety evaluation.

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 Animals Technology, and by attending appropriate training courses and conferences, or getting feedback from such events.

83. Zebrafish models for investigating cancer cells migration, invasion and 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, Cell migration, Cell invasion, Metastasis, Zebrafish

Animal types	Life stages
Zebra fish (Danio rerio)	embryo, neonate, juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this process is to characterise how cancer cells migrate through tissues, to identify novel potential drug targets to limit the formation of cancer 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?

Metastasis is the biggest life-threatening event for cancer patients. It is a process through which cancer cells can detach from a tumour and reach other locations in the body. Currently, about 90% of cancer-related deaths are caused by metastasis. Cancer cells need to become motile to be able to form metastasis and this process is still poorly understood. The knowledge generated by this project will lead the way to the development

of novel therapeutic strategies to improve the survival of breast, ovarian and pancreatic cancer patients.

What outputs do you think you will see at the end of this project?

This research will reveal the mechanisms responsible for the movement of cancer cells away from a primary tumour and whether this can be targeted by drugs to prevent the spreading of cancer cells throughout the body. It will advance our knowledge of how cell migration is regulated in cancer and this could lead to the discovery of new strategies for treating breast, ovarian and pancreatic cancer. We will publish the results from this project in peer-reviewed articles, as well as by posting pre-prints on open access servers, such as bioRxiv. This means that our results will be timely available to the scientific community. In addition, we will present the results in national and international conferences, in the form of posters and presentations. Key highlights from this work will be shared through the lab social media accounts (Facebook, Twitter and Instagram).

Who or what will benefit from these outputs, and how?

in the short term, these outputs will benefit researchers working on cancer biology, as similar approaches could be applied to different cancer types not considered in this work. In the long term, this work will inform further in vivo research using mammalian systems (mice), and eventually cancer patients.

How will you look to maximise the outputs of this work?

We will maximise the outputs of this work through the publication of the results in open-access journals and pre-print servers. We intend to include both successful and unsuccessful approaches. We will also publish methods papers, to make our approach available to other researchers interested in using Zebrafish as a model of metastasis. In addition, we will present the work at scientific meetings, where we will also share techniques utilised during the project.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 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.

Zebrafish is highly appropriate for modelling cancer and investigating therapeutic efficacy. Indeed, cells from liver, colon, pancreas, prostate, lung, ovary and breast cancers have been implanted into Zebrafish (Chen et al., 2021). These fish are easy to take care of, which makes them simple to maintain in a laboratory setting. They can be bred quickly, allowing the generation of large numbers of animals in a relatively short amount of time. Their offspring develop outside of the mother's body, which makes embryo manipulation for experiments easier. Finally, there is a high degree of similarity between zebrafish and humans, which make them a good model to study human disease, including drug development. We have chosen the larval stage as it is the most appropriate to perform live

cell imaging, allowing clear visualisation and quantification of cancer cell migration. Larval stages are the simplest vertebrate models that are suitable for such studies.

Typically, what will be done to an animal used in your project?

Approximately 400 cancer cells will be injected into 2 day post-fertilisation embryos. A few hours after injection, the embryos will be checked to identify damage induced by the injections. If these are detected, the embryos will be killed. Embryos will be monitored every day and grown reaching a maximum of 17dpf, to allow cancer cell dissemination through the tissues. Some embryos will be treated with potential anti-metastatic drugs. Live cell imaging will be performed throughout, during which time the larvae will be anaesthetised and immobilised, and all the animals will be killed at the end of the experiment for downstream analysis. In pilot experiments, the same larvae will be imaged multiple times to identify the best time point to visualise cancer cell dissemination. Once this has been determined, larvae will be mostly imaged once at end point (AC).

What are the expected impacts and/or adverse effects for the animals during your project?

Injection of tumour cells may rarely cause minor trauma which should heal rapidly (within hours). Transplanted cancer cells might produce adverse effects such as pain and physical debilitation. Fish will be monitored frequently for signs of ill-health and killed (e.g. a Schedule 1 method) before tumour formation interferes significantly with feeding, locomotion, respiration or cardiovascular function, or causes significant behavioural abnormality.

Expected severity categories and the 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 is moderate for all the animals undergoing the experimental procedures described in protocol 1 (50%) and mild for the breeding of the animals described in protocol 2 (50%).

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Exploratory studies, where possible, are first performed in human cell lines or in zebrafish embryos. Data is also generated from human tumours. However, the involvement of

multiple cell types in the process of cancer formation and progression and treatment response is currently impossible to fully represent other than in a mature organism.

Throughout the duration of the project, we will seek, review and incorporate new alternatives, wherever possible.

Which non-animal alternatives did you consider for use in this project?

We have already performed extensive studies in cell culture systems, using a variety of cell lines and a combination of simple and more complex 3-dimensional models (based on pubmed searches for "in vitro metastasis models", "3D metastasis models", "3D metastasis in vitro"; >1,000 results, mostly using extracellular matrices or scaffolds, with a single cancer cell line. More recently, tumour-on-a-chip models have been developed, reviewed by Bao Xian Huang and Tu 2023, but they failed to recapitulate the long distance migration often observed during metastasis in vivo). In addition, we have performed exploratory studies in non-protected zebrafish embryos (<5 days after fertilisation). These were extremely useful to optimise our experimental protocols.

Why were they not suitable?

The cell culture models do not allow to fully reproduce the complexity of tumours in patients. In addition, the limited amount of time available in experiment carried out with non-protected embryos was not enough to fully recapitulate the metastatic process, only allowing us to assess very early stages of the process.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We used the law of diminishing return (Mead's resource equation) to calculate the numbers of animals needed for our pilot experiment, resulting in 10 animals per group. The pilot study will be used to collect the mean and standard deviation of the spread of tumour cells outside the injection site for each group. The difference in the means will be used to calculate the effect size for the power calculation.
The standard deviations, direction of effect (one or two-tailed test) and the attrition rate (fish lost during the experiment) will also be taken into account in the power calculation.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We discussed our approach with a Senior Statistical Consultant at the Statistical Services Unit. Our initial calculations determined 10 animals per group in pilot experiments, where we will compare the behaviour of cancer cells with normal epithelial cells. We expect to see cancer cells spreading away from the injection site, while the normal cells will remained confined in the injection area. We plan to use a one-tail T test to analyse our

data. From this results, we will perform power calculation to refine the numbers in following experiments.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Pilot experiments will be used to optimise the number of animals to use. In addition, the ability to image tumour development in the same fish at repeated time points reduces the overall number of animals required.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Zebrafish have a simpler brain compared to rodents, with fewer neurons and less complex neuronal circuits (Mueller and Wullimann, 2016), suggesting a reduced awareness and pain sensation compared to mice. Furthermore, they have remarkable abilities to regenerate damaged tissues combined with potent antimicrobial activity in mucus secretions. For these reasons they arguably suffer less than mice for the same disease burden or intervention. Zebrafish will be humanely killed as soon as tumour formation and metastasis are sufficient to yield the desired data (i.e. differences in cancer cell dissemination), which will be long before interventions can interfere significantly with feeding, locomotion, respiration or cardiovascular function, or induce significant behavioural or other physiological abnormality. Any potential suffering will be mitigated by frequent inspection and early intervention. Regarding exposure to experimental drugs, small scale pilot experiments will first be conducted to establish safety, before expanding numbers. The zebrafish are housed in a purpose-built aquarium that is maintained by dedicated and skilled staff. A vet is also available to advise on zebrafish welfare.

Why can’t you use animals that are less sentient?

Mice are currently the most widely used model to study cancer metastasis (Hebert et al., 2023). Exploratory studies were performed in non-protected zebrafish embryos. These were extremely useful to optimise our experimental protocols. However, the limited amount of time available in these settings was not enough to fully recapitulate the metastatic process.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will regularly monitor the animals, daily after injection or intervention treatment. Should they present any sign of pain or ill-health, we will humanely kill them. All transplantations will be performed under anaesthesia. Our work will be supported by the experienced aquarium team. If necessary, we will seek advice from NVS and NACWO.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow PREPARE and ARRIVE guidelines

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will have regular discussions with the Named Persons and animal technicians to review current approaches and whether there are any new 3Rs opportunities. I will subscribe to the NC3Rs e- newsletter, to be up-to-date with NC3R events and publication. Where relevant, we will attend NC3R workshops and events.

84. Distributed information processing in neural circuits

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Circuit, Learning, Behaviour, Neuron, Synapse

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to investigate how distributed populations of neurons in the brain represent and transform sensory and motor information, distinguish sensory stimuli, extract features, learn sequences and coordinate movements. It will also elucidate the synaptic, dendritic and cellular properties that underpin these neural computations.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Understanding how neural circuits perform the computations required to make decisions, learn new tasks and generate complex motor behaviours is of fundamental importance in neuroscience and is a prerequisite for understanding many poorly understood neuronal disorders (e.g. schizophrenia and autism). We currently know very little about how different circuits in the brain represent and process the sensory (e.g. touch, vision) and motor (movement related) information required to carry out these functions. This research program is important as it will generate new scientific knowledge on how circuits in two major brain regions, the neocortex and cerebellum, combine and transform information

and work together to learn complex sensory and motor tasks. The physiological mechanisms involved span a wide range of spatial scales from molecular reactions to neuronal activity distributed across systems of networks. But how low level properties such as synapses and the processing carried out by individual neurons contribute to higher level processing is poorly understood. The basic research in this project aims to elucidate how these mechanisms underpin processing within neuronal populations in the neocortex and cerebellum during behaviour. This is key for developing a mechanistic understanding of sensory and motor processing in health and disease.

What outputs do you think you will see at the end of this project?

New scientific knowledge on sensorimotor processing

The main output of this research programme is to generate new scientific knowledge about how the activity across distributed populations of neurons in the neocortex, cerebellum and intermediate nuclei (the cortico-cerebellar system) represent and transform sensory and motor information, distinguish sensory stimuli, extract features, learn novel tasks and coordinate movements, since these core functions are poorly understood both in health and in disease. By testing current hypotheses and longstanding ideas of information processing in these circuits, this project will provide valuable new insights into cerebellar and neocortical function. This will include understanding how low level synaptic and cellular properties contribute to higher level coding across neuronal populations and how neural processing that is distributed across different brain regions works together as a system to form associations, learn motor tasks, predict the sensory consequences of movement and make decisions. Another key area will be to elucidate the neuronal computations performed as patterns of synaptic inputs activate the complex tree-like processes (dendrites) of neurons and are transformed into neuronal firing. These findings will provide a mechanistic understanding of information processing in these circuits and will also enable us to identify general principles that are applicable to other brain regions. Our discoveries will be actively disseminated through scientific conference presentations and publications in high-quality peer-reviewed journals.

New knowledge on the optical properties of brain tissue and the development of novel microscopes for high speed deep tissue imaging and photostimulation

During this research programme we will also generate new optical tools for imaging deeper within brain tissue and perturbing neurons and circuits in awake behaving animals. Through a collaborative project we will gain new knowledge on the optical properties of brain tissue and develop new microscopy methods and approaches to improve imaging at depth by compensating for the scattering of light as it passes through brain tissue by using special deformable mirrors. This will extend high spatiotemporal 3D functional imaging to deeper brain regions. These discoveries and technological developments will be actively disseminated to other laboratories, through scientific conference presentations, licensing to microscope manufactures and publications in high-quality peer-reviewed journals. Providing more powerful tools for studying brain function is an important output, as it will accelerate the pace of discovery in this important field. Moreover, by enabling better quality measurements of neural activity this new microscope technology is expected to reduce the number of animals required to address questions on information processing in neural circuits.

Neuroinformatic developments to enable sharing of computer models and experimental data

Development of a mechanistic understanding of information processing in neural circuits requires the construction and application of biologically detailed models of brain function. During this research programme we will use our experimental measurements to build mathematical models of interconnected neocortical and cerebellar circuits, enabling us to understand and predict how they work together as a whole. Such models are a valuable output because they generate sophisticated predictions of circuit function, generate new hypotheses, consolidate quantitative knowledge and enable a better understanding of complex neural systems. This knowledge will be shared through scientific conference presentations and through publications in high-quality peer-reviewed journals.

Moreover, the use of models to make physically plausible predictions and to explore a wider range of parameters than is feasible with experiments will enable greater refinement in experimental design and reduced animal usage.

Developing new data-driven neuronal and circuit models and the software tools used to create them are key endeavours for advancing our understanding of circuit function. Over the last decade we have developed extensive open source online resources and standards to collaboratively develop and share models in an accessible format for reuse. We are currently extending this platform to make it easier to build models and, importantly, to bring together and analyse the neurophysiological data on which they are based. This new online neuroinformatics infrastructure will enable our unique imaging and electrophysiological data sets and associated models of the cortico-cerebellar system (together with data and models from other labs around the world) to be made freely available to the scientific community in standardised accessible formats for reuse in future scientific studies that would otherwise require further animal experiments.

Who or what will benefit from these outputs, and how?

In the short term to medium term our work will generate a wealth of new data and knowledge on the functional properties of cerebellar and neocortical circuits and provide new insights into how neurons represent and transform sensorimotor information, extract features, learn new tasks and coordinate movement. The availability of this data and dissemination of our findings through peer-reviewed publication will benefit academics in the neuroscience research community and beyond. In addition, our work investigating light scattering and the optical properties of brain tissue will benefit academics in soft matter physics and provide new information for researchers throughout the broad field of biomedical optics. This project will also develop techniques and tools that will enable imaging deeper within the brain than is currently possible, providing transformative new tools for studying neural circuits and opening up new areas of the brain for investigation. This will benefit researchers working in diverse neuroscience fields.

Understanding how the brain learns new skills, represents and transforms information and the physiological mechanisms underlying these essential functions in healthy brains are valuable in their own right. This knowledge is also essential for understanding neurological disorders, such as epilepsy, schizophrenia and autism, which exhibit disruptions in and/or abnormalities of information processing. Unfortunately, it is presently difficult to design better treatments for neurological disorders because the function(s) of the networks affected are poorly understood. Developing an understanding of the healthy brain that links the low level properties to high level network function, will provide a framework for understanding how genetic and disease-induced changes in proteins, synapses and neurons cause aberrant network behaviour. Identifying neuromodulatory subsystems, key

neurotransmitter receptors and signalling pathways involved will all provide potential targets for intervention. Further, analysis of established disease models where alterations of proteins, synapses or neurons are predicted to change network behaviour by disrupting electrophysiological or synaptic properties will provide insight into how such disruptions contribute to disease specific phenotypes. According to the World Health Organization, disorders of the brain affect up to one in three people worldwide and the European Brain Council (www.europeanbraincouncil.org) has calculated that brain disorders cost Europe €800 billion every year with the UK alone spending an estimated £100 billion annually. Together these emphasise the need for fundamental research to better understand neural function in health and disease, which in the longer term, could lead to improved treatments.

Other beneficiaries include early stage researchers and students who will receive cross-discipline training in cutting edge neuroscience techniques including small animal surgery, electrophysiology, head-fixed imaging and optogenetic techniques and analysis and will create opportunities in career development during the project. Our work on the optimisation of forelimb reaching tasks in mice and other behavioural training paradigms will also provide benefit to the neuroscience community through the sharing and refinement of techniques with other groups.

How will you look to maximise the outputs of this work?

In order to maximise dissemination of knowledge from this project we will endeavour to actively share the outputs to as broad an audience as possible (neuroscientists, theoreticians, computational scientists, optical physicists, microscope and software developers, students, the pharmaceutical industry and clinicians). This will be achieved through scientific presentations and posters at local, regional and international conferences throughout the course of the project and through publications in high-quality peer-reviewed journals in a range of subject areas such as experimental and computational neuroscience, physiology, computational biology, biophysics, microscopy, optical physics, and neuroinformatics. Null results are always considered to be an important part of the scientific investigation and where appropriate these are included and discussed within our publications.

In order to maximise accessibility to the experimental output from this project, experimental data will be made publicly available through the data sharing repository Figshare and linked to the publication.

Analysis scripts will be made available through the code sharing repository GitHub. Researchers are expected to structure their data, analysis and documents to be compatible with data repositories and the ‘FAIR’ principles (Findable, Accessible, Interoperable and Reproducible). Enabling data sharing is a key factor in the design requirements of our microscope datafiles (which include headers with extensive metadata on the experimental details) and analysis pipelines, and we will publish datasets with accompanying documentation detailing experimental conditions and analysis scripts to regenerate figures.

Several developments that will be completed in the next few years will augment sharing of data and models during the project. Firstly, we are developing and promoting standards for data and model sharing. Indeed we are organising a week-long international meeting and hackathon to bring together world experts on data sharing and help researchers convert their data into standardised formats, which enables complex heterogeneous data types to

be combined. In addition, our development of an open online resource for model building and analysing data will greatly facilitate dissemination of the experimental data used to build and test our models of the cortico-cerebellar system. This will enable anyone with the internet to access the data, analysis and models we produce.

To maximise the outputs from our work on the development of optical tools for deep tissue imaging of neurons and circuits in awake behaving animals we will actively disseminate novel microscope designs through publications, web resources and LabVIEW and MatLab versions of our microscope software will also be open source and made available on GitHub as they are developed during the project.

We have also formed extensive collaborations within our own institution, throughout the UK and internationally with other institutions across the world including in the US and the EU. These collaborations enable us to maximise the output of our work by both sharing and utilising the latest developments in imaging technology, viral tools and experimental strategies, thereby increasing the impact of our work on the field through the integration of efforts between groups to answer important biological questions across many fields.

Species and numbers of animals expected to be used

- Mice: 5000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are the most appropriate species for this project because they are mammals and have cortical and cerebellar networks with largely similar structure to humans. The basic synaptic, neuronal and network properties found in mice make them a good model system for studying human brain function and mice are the least sentient species that are appropriate for this type of study. Importantly, the project relies on transgenic mouse technology to genetically label identified neuronal populations and specific cell types, which is only possible with utilisation of specific, well characterised mouse lines which restrict the gene expression or deletion to defined components of cortical and cerebellar networks. This technology is therefore critical for precisely targeted and inducible gene expression. In addition, this project builds on the considerable amount of information available on the electrophysiological and anatomical properties of neurons in the mouse neocortex and cerebellum and requires the use of motor and sensory behavioural paradigms that have been established and refined for this species.

We will primarily use adult mice since our study is on information processing in neural circuits in the mature nervous system, so recordings are carried out at the life stage beyond the embryonic and neonatal periods, which involve significant developmental changes. The use of adult mice is also important to accurately target specific brain regions, which are based on the 3D brain architecture of the adult mouse. In addition, our training protocols are also refined for use in adult animals where the growth curve of the animal has begun to plateau.

In order to genetically label specific cell types, we will also use expression strategies

involving mouse embryos, where the introduction of DNA vectors at defined embryonic stages enables precise targeting of cells which then migrate and mature into defined cell types of interest in the adult. For some experimental strategies, we will also need to introduce expression vectors into juvenile mice in order to enable an extended period of expression for later recordings in the mature adult or use juvenile mice for preparation of acute brain slices for optimal integrity and viability of the sliced tissue.

Typically, what will be done to an animal used in your project?

Typically, a wild type or transgenic mouse generated under a standard breeding protocol (Mild Severity) will undergo recovery surgery with general anaesthesia to deliver a DNA/Viral expression vector(s) to the mouse brain, either in utero to the developing mouse embryo or by stereotaxic guidance where the vector may be co-injected with microbeads into the juvenile or adult mouse (both Moderate Severity). A head fixation plate will subsequently be adhered to the skull of the adult mouse and a craniotomy performed to generate a cranial window, to implant optical elements for imaging or alternatively to implant microelectrodes for electrophysiological recordings. The microinjection and cranial implant surgeries are performed under general anaesthesia with recovery (Moderate Severity) and may be performed in a single operation or as two separate surgeries depending on the requirements of the expression strategy. Following a full recovery (minimum 1 week), typically the mouse is then trained in a behavioural paradigm (e.g. a forelimb or whisker task) whilst under head fixation and optical or electrophysiological recordings are made in the awake mouse under no anaesthesia. In order to motivate the mice to learn and perform these tasks, access to water is controlled and water is delivered during the task as a reward. Usually animals are trained and/or recorded daily for 1-3 hours over a period of 8-12 weeks. Recordings can include electrophysiological, pharmacological, optogenetic or chemogenetic manipulations and in some instances recordings are made under general anaesthesia with or without recovery. At the end of these procedures, mice typically undergo non-recovery terminal general anaesthesia where they are either transcardially perfused to preserve the brain for anatomical studies or immunolabeling or they are decapitated with a guillotine in order to prepare acute brain slices for electrophysiological recordings.

What are the expected impacts and/or adverse effects for the animals during your project?

Transgenic mice that have been generated through standard breeding protocols to provide mice for this project are without a harmful phenotype and are not expected to show adverse effects.

Mice that have undergone laparotomy for in utero electroporation, microinjection or cranial window/headplate implant surgery with good post-operative care, including effective analgesia are likely to experience short-term moderate pain. They are expected to have returned essentially to normal within 48-72 hours but may show initial weight loss post surgery. Other potential adverse effects related to the surgical procedures include a poor recovery from anaesthesia, an infection or skin irritation post surgery or the possibility of a detrimental phenotype arising from damage to neural tissue. All of these are uncommon. Typically, animals will experience mild, transient pain and no lasting harm from administration of substances by injection using standard routes (subcutaneous, intraperitoneal).

Mice that are under water restriction protocols for training on a behavioural task may show

early signs of dehydration but receive immediate treatment appropriate for the clinical signs so this is considered short term. Animals may also show initial signs of stress to head fixation or behavioural training which lessens with familiarisation.

Electrophysiological, pharmacological, optogenetic or chemogenetic manipulation of neuronal activity during in vivo recordings has the potential to result in unexpected adverse reactions such as behavioural seizures but this is highly improbable given our sparse and local expression strategies.

The terminal procedures will be carried out under non-recovery anaesthesia where the animals will only be aware of the anaesthetic being administered and may experience mild distress and no pain.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The phenotype(s) of transgenic mice generated through standard breeding protocols on this project are expected to be sub-threshold as the mice are not expected to manifest any adverse effects.
Genotyping will generally be undertaken using surplus material from ear notching for identification and the prospective severity limit for this protocol will be Mild. Mice that have undergone surgery with good post-operative care, including effective analgesia are likely to experience short-term moderate pain and are expected to have returned essentially to normal within 48-72 hours. A moderate severity classification is appropriate for such procedures. Mice under water restriction protocols are not expected to experience significant impairment in the well-being or general condition of the animal (mild). The majority of recordings of neural activity under head fixation are anticipated to be sub-threshold as the mice are not expected to manifest any adverse effects. For those that are imaged under anaesthesia with recovery, a moderate severity limit will apply.

We anticipate that the majority of our experiments (approximately 70%) will involve surgical procedures for the delivery of expression vectors/microbeads and for cranial implants or windows and that the subsequent procedures that these mice undergo (water restriction, task training, recordings under head fixation or experiments under terminal anaesthesia) will have a lower severity classification. We do not expect these techniques to be cumulative, with mice returning essentially back to normal between steps and with no increasing impact or sensitisation, the overall experience of the majority of animals will be moderate in severity.

The remainder of our experiments (approximately 30%) will involve only tissue preparation from transgenic or wild type animals in non-recovery procedures (AC), where level of anaesthesia is sufficient for the animal to feel no pain. For these mice the overall experience would have a severity limit of mild.

Although we endeavour to minimise any surplus breeding from our transgenic colonies, we anticipate that a proportion of our total mice bred will be surplus to experiments (due to specific age or genotype requirements). These mice and those used solely for breeding to maintain our transgenic colonies are expected to have an overall experience of sub-threshold. They are not expected to manifest any adverse effect and genotyping will

generally be undertaken using surplus material from ear notching for identification.

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?

Experiments on live brain tissue and intact behaving animals are essential for gaining new knowledge on properties of synaptic transmission, neuronal computation and circuit function and there is no alternative to using animals. Moreover, investigations examining how information is represented in the activity of populations of neurons and conveyed between different structures in the brain require recordings of neural activity in a fully intact, live behaving animal.

Which non-animal alternatives did you consider for use in this project?

Humans, animals with less sentience, terminal experiments, other non-protected species, neuronal cell culture, brain organoids, and computer modelling of neuronal and network function were considered.

Why were they not suitable?

Human subjects are not suitable for this research project because the recording techniques required are invasive, and the experiments require transgenic technologies for the expression of optical reporters and transducers. It is therefore not possible to manipulate and record neural activity in the cortico-cerebellar system in humans during behavioural tasks.

Less sentient or non-protected species are not suitable since the project requires animal subjects that have cortical and cerebellar networks with largely similar structure to humans that are able to learn and execute complex sensory discrimination motor tasks and behaviour. The use of animals at a more immature life stage with less sentience would not be possible as the developing brain has vastly different brain architecture and connectivity and as with the use of anaesthetised animals in terminal experiments would not be compatible with the performance of behavioural tasks. In addition, anaesthesia is known to interfere with the physiological processes under study.

Cell culture and brain organoids are not suitable because synaptic, neuronal and network properties all change in such preparations, they do not have the architecture, connectivity, neuromodulation or functionality of the intact brain, but most importantly, they do not permit investigation into how behavioural information is represented, processed and learned in neural populations. Whilst computer modelling of neuronal and network function is a useful tool to explore complex interactions between variables once they are known, it is fundamentally limited by the knowledge base on which the models are built. This project requires the use of animals as the functional properties and physiological parameters

under study are not known and require recordings of neural activity in fully intact, live behaving mice and there are currently no computer models or equivalent that can accurately and effectively model these phenomena. It should be noted that our project will involve generating computer models of the circuits under study in order to better understand information processing in complex networks, test the plausibility of different ideas and to generate more informed predictions that are experimentally testable, thereby enabling better focused experimental protocols.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers of animals required and the experimental strategies proposed are built on related studies that we have performed over the past 5 years. The design has been based on studies published in peer reviewed scientific journals and has incorporated guidance on experimental design from online and workshop resources from the National Centre for the Replacement Refinement & Reduction of Animals in Research (NC3Rs).

The numbers of animals used in this project will also be reviewed annually as part of our Ethical Review Process.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

To plan our experimental design and practical methodology we have utilised the guidance on experimental design from online and workshop resources from the National Centre for the Replacement, Refinement & Reduction of Animals in Research (NC3Rs). We have also used NC3Rs guidance for sample size calculations and the use of appropriate statistical analysis. We apply methods of experimental design which enable us to estimate the minimum number of animals required to detect an effect and each experiment is designed, as far as possible, to include its own control, reducing variability, increasing statistical sensitivity and thus minimising the number of animals required to reach the required level of confidence. Variability across animals is also minimised by using well defined inbred strains of mice, with little genetic variation.

We have carefully considered the behavioural tasks and training methodologies to employ to ensure the experiments provide high-yield data that are highly specific to the parameters under investigation and optimal for achieving the necessary statistical power through the generation of large numbers of highly repeatable trials. We assay multiple behavioural parameters during task performance, and to minimise the impact of task-unrelated activity, we analyse single trial covariability and trial-averaged task-related activation. During the planning of our experiments, we also consider the selection of the most appropriate genetic tool for the parameter under study, for example the use of different biosensor variants with different kinetics, enable us to increase the precision of our measurements.

We also carefully consider the experimental preparation that we use in order to maximise data collection from the minimum number of animals. Multiple recordings can be achieved from one animal and chronic preparations enable repeat imaging, thereby reducing the number of animals required to address key questions.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Where appropriate, we utilise techniques such as viral transfection and in utero electroporation for targeted delivery of genetic material as this reduces the need to keep large transgenic colonies which require breeding and maintenance over several generations.

Experimental animal requirements are planned far in advance so that my lab manager/technician can micromanage efficient breeding and ensure that the number of animals bred matches, as closely as possible, the experimental requirements. We apply the most efficient breeding strategies and where possible will keep transgenic lines in a homozygous rather than heterozygous state in order to reduce the numbers of non-transgenic offspring generated from crosses which cannot be used for experiments (e.g. for fluorescent reporter transgenic mouse lines).

Cryopreservation of transgenic lines is also utilised whenever there is a sustained gap in experimental use in order to minimise breeding surplus.

When a surplus exists we closely coordinate with neighbouring groups to enable sharing of tissue where possible and we are registered users of a local animal and tissue share exchange enabling researchers to share or request surplus animals or tissue, thereby reducing the number of animals being used.
We perform pilot studies to test viral constructs for new biosensors or chemogenetic/optogenetic tools in order to determine suitability before scaling up for full experimental use. In some instances, we have found that it is possible to test multiple constructs within a single animal by microinjection into adjacent cerebellar lobules thereby reducing the number of mice used in testing new viral tools.

Wherever possible we also develop models to explore complex interactions between variables and make predictions. This allows us to reduce animal usage by designing better focused experimental protocols for these complex, high-dimensional biological systems.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 least sentient species that are appropriate for this type of study. Importantly, the project relies on the use of transgenic

mouse technology to genetically label neuronal populations and specific cell types in well characterised mouse lines. The lines we select show no aversive phenotypes and are not expected to cause any suffering to the animals.

Our surgical methods for the delivery of viral tools, for cranial windows and the attachment of headplates/implants in mice are based on highly advanced surgical techniques which have been established and refined for this species for many years. We rely on the high resolution stereotaxic maps of the mouse brain to enable us to precisely target the brain regions under study and we also utilise extensive research on developmental stages in the mouse embryo in order to target specific cell types. Suffering, distress, or lasting harm from surgery is minimised using the most appropriate anaesthesia and analgesia which is under regular review and ensuring sufficient depth of anaesthesia is maintained throughout the surgery. We also use highly specialised surgical technique, precision microsurgery tools and specialised headplate designs to minimise any complications from surgery.

Mice that undergo surgery are closely monitored using a post-surgical monitoring score sheet to rapidly identify any relevant clinical indicators. We also regularly review our surgical methods to incorporate advances to improve efficiency and to minimise adverse effects from surgery.

Our behavioural training techniques involve water restriction and reward and are based on standards in the field and established protocols that have been refined specifically for mice. We ensure the minimum restriction that provides the appropriate motivation required for the behavioural task is applied and our daily health assessment scoring ensures that any animal exhibiting adverse effects from the water restriction promptly receives additional water or is removed entirely from restriction.

Motor and sensory behavioural paradigms that we employ also consider the animals natural behavioural repertoire (e.g. forelimb and whisker tasks) and so require shorter training periods and less fluid control for motivation to perform these tasks. As our behavioural paradigms and imaging experiments involve head-fixation we employ refinements to minimise stress caused to the animal. This includes using a staged habituation to head-fixation involving familiarisation to the equipment and experimental setup and also ensuring they can adopt as natural/comfortable position as possible during the fixation and keeping any sensory stimulation within natural levels.

For our terminal experiments used for tissue preparation, we maintain the level of anaesthesia at sufficient depth for the animal to feel no pain.

Why can’t you use animals that are less sentient?

In order to study how the brain represents and processes sensory and motor information we require a model system that is able to learn and execute complex sensory discrimination and motor tasks and behaviours. The mouse represents the least sentient species that can be used. They are mammals and have cortical and cerebellar networks with largely similar structure to humans and are the most appropriate species for a study of this type. The use of a less sentient species such as the zebrafish would not be possible due to the significant differences in brain structure and function as they have a much reduced cerebellum and lack a neocortex.

The use of animals at a more immature life stage with less sentience would not be

possible as embryonic or newborn mice have vastly different brain architecture, network properties and connectivity and as in the use of anaesthetised animals, would not be compatible with the performance of behavioural tasks. In addition, anaesthesia is known to interfere with the physiological processes under study.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The procedures detailed in this licence are well established within our lab and we have developed several refinements in the protocols to minimise the welfare costs to the animals.

For our surgery, we utilise advanced surgical methods which incorporate techniques to minimise complications. This includes the use of microsurgery tools, specialised headplate designs and implants which are designed to be as small and unobtrusive as possible in order to minimise any damage to neural tissue. Our anaesthesia and analgesia regimen are under regular review. For example, we have extended the minimum duration of post surgery analgesia from 48 to 72 hours from our previous project licence. Mice that have undergone surgery are also closely monitored using a post-surgical monitoring sheet to rapidly identify any relevant clinical indicators.

For behavioural training, we have adopted refinements in order to minimise stress to the animal. This includes handling the animals using a tunnel or cupped hands which has been shown to decrease anxiety compared to handling by the tail and also by increasing the amount of time the animal experiences head fixation over a period of days. In addition, we configure our experimental setup to enable the animal to settle its spine into a natural posture and adopt a comfortable position of head relative to paws to reduce stress in the head-fixed set-up. We also closely monitor animals during behavioural tasks using high speed cameras.

Where water control is used for behavioural training, we employ the minimum restriction that provides the appropriate motivation required for the behavioural task. This is achieved by the refinement of absolute volumes based on ongoing analysis of task performance throughout the training. The motor and sensory behavioural paradigms that we employ also consider the animals natural behavioural repertoire (e.g. forelimb and whisker tasks) and so require shorter training periods and less fluid control for motivation to perform these tasks. We have also refined some of our experiments to incorporate breaks in fluid control into the design of the study which allow for a reduction in the time spent under water control. This also allows us to regularly establish new baseline ad libitum weights to ensure the accuracy of changes in body weight.

In addition, we will incorporate new refinements from the NC3R’s within our protocols. This will include a pre-op health screen where the mice will be carefully inspected before surgery or water restriction to detect any changes in health status or behaviour in order to exclude unsuitable animals from procedures. We will also deliver pre and/or post surgery fluid replacement for procedures lasting longer than an hour to maintain the hydration of the mice and also to aid recovery. For some experiments, we will also add sucrose, artificial sweetener or soya milk to the water reward as a refinement to make it more palatable and to increase motivation.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We conduct our experiments according to the PREPARE (Planning, Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines (Smith et al., 2018) and the ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines 2.0 (du Sert et al., 2020).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will remain informed of the latest 3Rs advances through NC3Rs workshops, newsletters and online resources, from peer reviewed publications, from attendance at our annual Biological Services Symposia and also through our AWERB Committee.

Our surgical methods, water restriction protocols, behavioural training and imaging protocols will undergo regular review throughout the duration of the project to identify and implement any new refinements and advances.

85. Recalibrating host responses to infectious disease to minimise tissue injury

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Bacteria, Inflammation, Host-based therapy, Antimicrobial resistance, Respiratory disease

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The project aims to identify immune responses to infections that can be manipulated to improve the clearance of micro-organisms that cause infectious diseases. The goal is to maximise clearance of these infections while minimising the unwanted consequences of the immune response that can result in severe disease. The focus is on bacterial pneumonia and on how chronic obstructive pulmonary disease impacts susceptibility and outcome of pneumonia but other infections at other sites and their relationship to factors influencing susceptibility will also be explored.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Infections, in particular pneumonia, remain a leading cause of human and animal mortality worldwide, impacting the poorest areas of society disproportionally. Most people are frequently exposed to micro- organisms such as bacteria and viruses, that have the potential to cause severe disease but usually do not do so because of effective immune responses. People who develop serious illness are the exception and do so because essential immune responses are less effective. These individuals then require antibiotics to treat infections, but the effectiveness of this approach is threatened by increasing resistance to antibiotics by micro-organisms. In addition, susceptible infections respond incompletely in the most vulnerable individuals since management focuses on pathogen clearance but does not address the inflammatory response that is set in motion by infection. We wish to reduce reliance on antibiotics and improve outcomes of treatment of infections by developing strategies that recalibrate the individual's immune response when they are susceptible to severe infection so that it resembles the majority of people who able to withstand infection.

What outputs do you think you will see at the end of this project?

The goal of this programme is to identify pathways and therapeutic strategies that enhance the clearance of micro-organisms that cause infections, called pathogens, and minimise unwanted consequences of harmful inflammatory responses generated by these pathogens. A secondary objective is to better understand the basis of susceptibility to infection in terms of how immune cells termed macrophages and neutrophils clear pathogens and the regulation of the inflammatory response that if excessive can lead to manifestations of severe disease. We will identify key responses to pathogens that underpin resilience to infection and identify how certain epidemiological factors associated with disease susceptibility alter these responses. The major focus will be on bacterial pneumonia and the primary disease association will be how features associated with chronic obstructive pulmonary disease (COPD), a common smoking related chronic lung disease, alter these responses in macrophages and neutrophils. A particular focus is on how alterations in cell metabolism, the process by which cells burn fuel to generate energy, and how the cell organelles involved in generating energy for cells, termed mitochondria, alter their responses to infection in health and COPD. Outputs will be in the short-term through scientific discovery presented at research meetings or published in scientific literature and in the medium-term through development of therapeutic strategies, including with industry partners, which may lead to drugs that can be tested in future clinical trials in humans.

Who or what will benefit from these outputs, and how?

The short-term beneficiaries will be the scientific community in particular those working in the fields of immunology, infection and respiratory disease. Benefits will be scientific and will include development of models that replicate aspects of human disease with fidelity.

Medium term beneficiaries will include clinical researchers and industry partners who may be able to understand more about disease processes or therapeutic strategies that can inform development of approaches to investigate or treat patients at increased risk of infectious disease.

The long-term beneficiaries will be humans and animals who can benefit from new therapeutic approaches with less reliance on antimicrobials. This will limit the potential

complications of antimicrobial resistant micro-organisms and allow more personalised approaches to management of infectious diseases.

How will you look to maximise the outputs of this work?

Outputs will be achieved through:

Scientific publications and meeting presentations sharing discoveries, open access data sets and methodologies

Collaborations adding value to other academic activities and by sharing information with industry stakeholders to help progress development of therapies

Engagement with funders and professional bodies to highlight scientific needs to help advance the field Sharing assets with clinical trialists to aid clinical translation

Species and numbers of animals expected to be used

- Mice: 11,250

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

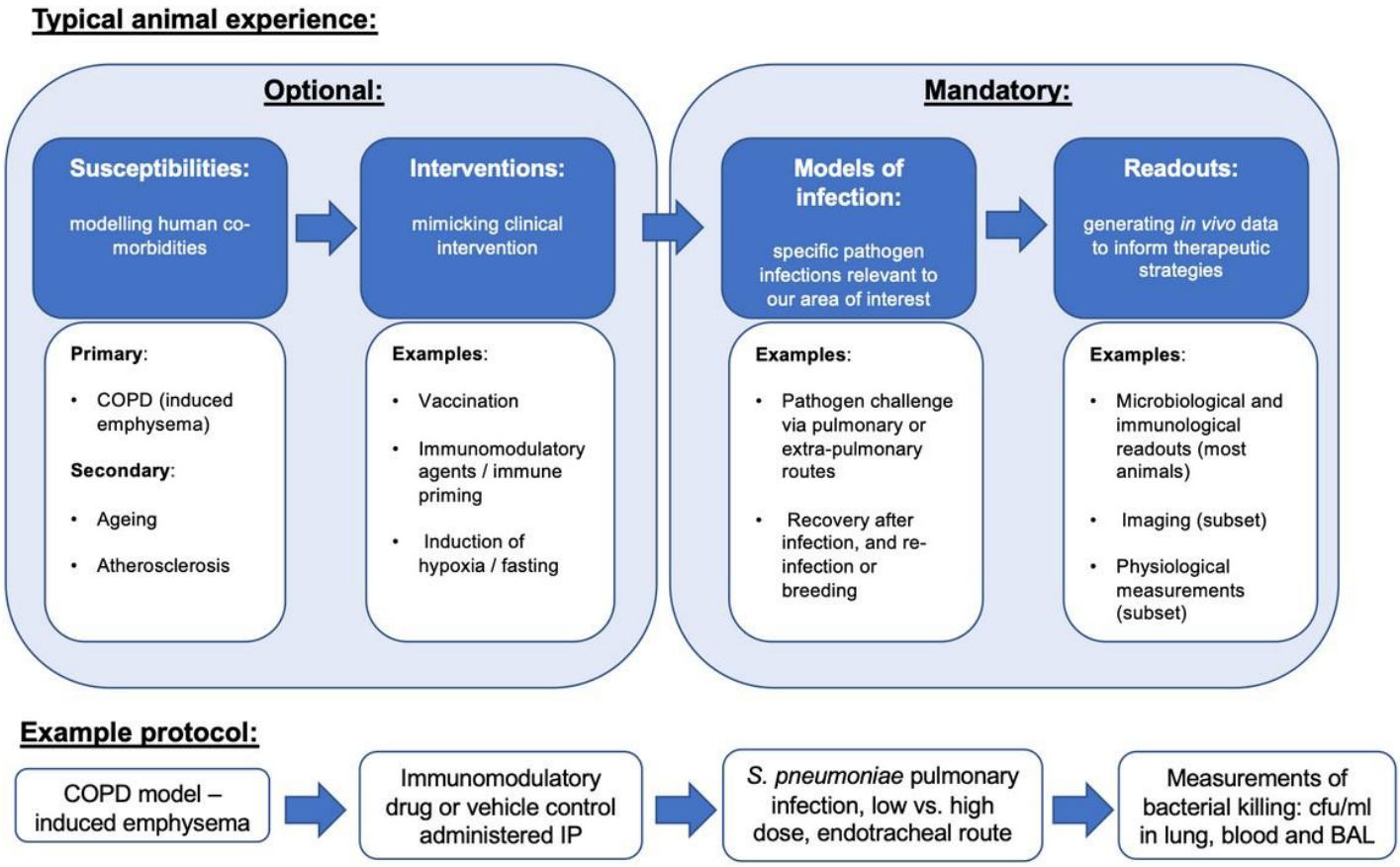
Instigation of host responses to bacteria and regulation of inflammatory responses requires complex models with multiple cell types. While mechanisms can be initially studied in tissue models and we increasingly used multi-cellular tissue models in assessment, findings require validation in models that contain all relevant cell types working in combination. Mice are an appropriate model since their immune system, inflammatory responses (including in the lung) and responses to many pathogens replicate humans. A range of assays to study inflammation, genetically modified mice and data on drug dosing aid their adoption for studies on lung and also extra-pulmonary inflammation and response to infection. Whilst no single animal model represents the complexity of diseases such as COPD, we are able to use models that cause emphysema and susceptibility to COPD pathogens, while some of our genetically modified mouse strains have similar immune cell (e.g. macrophage) defects as those seen in COPD.

We continue to evaluate alternative non-mammalian models, e.g. zebrafish to image key cellular structures such as the mitochondria, but mice remain important for studies of infection and inflammation, particularly in the lung.

We will predominantly use adult mice. Occasionally where an intervention acts at an earlier stage of development to modify the immune response we may test an intervention in young mice. For example interventions that shape innate immune development, such as alterations in the microbiome, may need to be tested when the innate immune system is still maturing in early life.

Typically, what will be done to an animal used in your project?

The basic model is to instil a pathogen in the lung or at an alternative extra-pulmonary site and to record the number of viable pathogens (bacteria or viruses), the inflammatory response (cells and proteins) and physiological responses (e.g. weight) at set time points typically from 4 hours up to 4 days after infection. In some experiments we will compare these results to a sham infection control relevant to the pathogen in question, e.g. using PBS or culture media. In some experiments we may modify the response to infection by creating a model of human disease (e.g. creating emphysema) before infection, by causing a prior viral infection, by priming the immune response (e.g. with microbial products or alteration in the microbiome), by treating with a drug or vaccine or by manipulating key cells involved in the response. In some experiments we may manipulate the response to infection with drugs or other interventions. Occasionally we will let the mice recover from infection and then determine how they or their off-spring respond to subsequent challenge.



What are the expected impacts and/or adverse effects for the animals during your project?

Our models primarily explore effective responses that result in pathogen clearance without disease or with only the early stages of mild disease. Some infection models, for example influenza A viral infection, more significant bacterial infection resulting in significant neutrophilic inflammation with bacteraemia or interventions to modulate susceptibility that increase disease from sub-clinical or mild stages can result in moderate severity disease but these are the exception. In these cases, signs may include some weight loss (e.g. influenza A virus) or some reduction in activity level. Mice are monitored regularly and are killed if any signs of more significant disease become apparent but these are not usual in these models. Models are typically run for 1-2 days after infection and very rarely for longer periods. The exception are the models in which we allow mice recover from an initial infection. The fact that mice recover and can go on to be rechallenged or to breed demonstrates that these models are well tolerated in most cases. Occasional procedures

involve a minor surgical procedure to insert monitoring equipment or a procedure to create a local infection such as skin injection. In these cases there may be minor degrees of local irritation at the procedure site.

In occasional cases mice are irradiated to deplete immune cells from their bone marrow, and are then given healthy stem cells from donor mice to reconstitute their blood-derived immune cells. This can also involve the use of shielding to protect tissue immune cells. Without reconstitution of blood immune cell populations this procedure is lethal and during 'engraftment' mice are at increased risk of infection and their environment must be carefully controlled to minimise the risk of infection. The dose of irradiation is required to prevent rejection of the graft by the original immune cells (which would make the mice very unwell). If, however, reconstitution with haematopoietic stem cells is successful, the irradiated mice recover well. This is a well-established procedure in the field and helps prove which cells mediate key responses. In the experience of our collaborators, deaths following irradiation and reconstitution are extremely rare (<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)?

Our models are graded as moderate in approximately 40% of cases and mild in the rest. Our experimental infection models are generally sub-clinical and cause no or only mild symptoms. Approximately one third will result in an increased degree of infection. All are monitored intensively to assess any signs of increased severity, particularly when manipulating host responses. Emphysema models are graded as moderate severity as is the adoptive transfer of bone marrow.

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?

Analysis of infection and inflammation requires investigation of how multiple types of cells work in co- operation. Using mice as a whole organism allows the analysis of responses to infection and regulation of inflammation in a multi-cellular system in which responses integrate responses from multiple different tissues that have been subject to physiological development in the same multi-cellular system. The use of genetically altered mice allows us to interrogate the function of particular genes in immune cells and other cells of interest. The availability of multiple tools to characterise immune responses and inflammation allows in depth analysis. Moreover, pharmacological approaches have been developed to allow testing of potential therapeutic approaches in mice. The in-depth characterisation of mouse infection and inflammation models and knowledge of similarities to humans mean results can be readily translated to man.

Which non-animal alternatives did you consider for use in this project?

Isolated (human) immune cells e.g. macrophages and neutrophils and co-cultures of immune cells with epithelial cells. Tissue models such as 3D models incorporating immune cells and induced pluripotent stem cell derived tissue organoids e.g. lung organoids.

Why were they not suitable?

Single cell or co-culture models are used to test initial findings. Some validation can be achieved in the more complex tissue culture models but these still do not include all relevant cell types. The multi- cellular interactions of a living organism are not yet amenable to investigation solely with alternatives to living organism and investigation of responses to therapeutic interventions still requires some testing in a living organism.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Data from our previous experiments and those of our collaborators are used to inform estimations relating to microbiological and inflammatory outcomes using values for colony forming units of bacteria, cells numbers in bronchoalveolar lavage fluid or cytokine levels. Where we have not previously conducted experiments (e.g. using a new immunomodulatory compound) we have factored in numbers of animals to conduct small pilot studies. Published literature is also used to inform new experiments.

Experiments involve multiple time points and each experimental condition needs control groups, involving sham infection and controls for a genetic line or a pharmacological intervention. Breeding and rederivation of lines also adds numbers to the total.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

For those experiments that analyse inflammation in vivo, we perform pilot studies prior to designing definitive experiments involving larger cohorts; experiments will be planned, conducted and reported according to NC3R and ARRIVE guidelines.

We minimise numbers of mice by collecting the maximum samples from individual mice, by the use of new assays, such as imaging based approaches, which enable the kinetics of responses to be measured in single mice and by refining assays in terms of sample volume and variability. Where possible we pool controls to reduce numbers and where possible will use congenic lines. A further step we are evaluating is the use of a new model of murine alveolar macrophages in which foetal liver cells are differentiated ex vivo into a cell line which can be expanded ex vivo and frozen down for future experiments. If evaluation shows this to be a reproducible model it will largely replace the need to isolate bone marrow-derived macrophages from mice killed by schedule 1 methods.

Before each experiment is conducted, a detailed protocol is written covering: (i) a statement of the experimental objectives; (ii) a description of the experiment, covering such matters as the experimental treatments, the size of the experiment, and the experimental material; and (iii) an outline of the method of analysis of the results. Factorial designs are preferred, and power analysis is used where appropriate. The animal facility is consulted during the development of these and they will be produced in line with PREPARE guidelines (https://norecopa.no/prepare/ prepare-checklist).

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We actively manage colonies reviewing breeding with animal facility staff to ensure numbers are kept to the appropriate levels for upcoming experiments. Where possible mice are bred in-house - this enables tighter control of relevant factors such as the microbiome, reducing experimental variation.

Pilot studies inform group sizes when new models are undertaken.

Multiple samples are taken from individual mice as above reducing total numbers.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We use mouse models of infection, predominantly bacterial or viral infection of the lung and occasionally at extra-pulmonary sites. Our models study the early stages of the response to infection and the resultant inflammatory response. The majority of models study the first 1-2 days after infection minimising the time mice are exposed to infection. Most models are of low dose challenge studying what protects against the kind of bacterial exposure that happens regularly to all living organisms. This means changes to this innate level of protection can result in low levels of infection versus no infection, or more marked inflammation in the early stages of disease before mice become unwell. Although some models involve infection and recovery these are designed to have low dose initial challenge of pathogen and may involve administration of antibacterial agents to reduce any impact of the infection. We have over twenty years refined the doses and strains used to accurately predict the tipping points for key immune responses we study giving precision in outcomes.

Why can’t you use animals that are less sentient?

Although different to humans, mice represent at present the best available animal model of infection and inflammation (particularly of the lung). Mouse models are representative and extensively characterised, in relation to human responses. Not only do mice have comparable immune system and inflammatory responses to humans, there is also a

wealth of genetic mutants and tools with which to analyse responses. In addition, there is a wealth of data on drug dosing enabling testing of pharmacological interventions. We have access to less sentient models, for example through collaboration we are exploring greater use of zebrafish models, particularly when genes of interest may have profound developmental consequences if modified. However, the mouse models remain an important component of evaluation of key host responses to infection.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Our infection and inflammatory models are mostly of short duration or involve low dose infections with recovery. This minimises suffering. doses are calculated based on multiple iterations of refinement to minimise suffering. where new infections or interventions are used initial dose finding experiments are performed to ensure minimal suffering and that infections are not of greater severity than anticipated.

We have developed systems of sub-clinical infection and developed sensitive assays to analyse the early stages of the inflammatory response to minimise suffering. Oropharyngeal instillation allows us to deliver small numbers of bacteria to the airway to mimic human disease with less variability than intranasal instillation. In earlier licences we used intratracheal instillation, which requires an anaesthetic and surgical incision. Although this provided accurate doses for infections, therefore decreasing our experimental variation and we have a large data set defining “tipping -points” for the transition from macrophage controlled sub-clinical infections to mild pneumonia we have replaced this procedure. We may on occasions need to perform some experiments by endotracheal or intratracheal routes but will only do this after consultation, where specifically requested to by funders or reviewers, so that we do not have to perform a large number of experiments to re-define tipping points or specific conditions with the oropharyngeal model. We will therefore use the oropharyngeal route for the majority of lung infections and occasionally use the endotracheal route.

We have refined our assessment of mice post procedure. Mice are regularly monitored (e.g. 1PM, 5PM, 9PM and 9AM after infections) and are killed by humane means if they show signs of significant physical distress (assessed on the basis of temperature, weight loss and a visual assessment score of activity level, response to handling, posture, piloerection, behaviour in a new setting and breathing).

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We stay up to date on best practice guidelines and updates by making use of the NC3Rs website (www.nc3rs.org.uk)

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We make use of the online resources provided by NC3R and follow developments in the literature. We moreover keep informed on new developments with the help of informational seminars and events held locally through our animal facility, and where applicable change our practice accordingly.

86. Evaluating the regenerative potential of biomaterials for skin wound repair

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Skin, Tissue engineering, Biomaterials, Immunomodulation, Wound closure therapy

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aims of the project are to develop and test biomaterials that can repair full-thickness skin wounds. Our rationale is centred on the urgent need to develop a new class of biomaterials, which encompass a range of synthetic or natural materials combined with cells or/and substances. These biomaterials aim to replace damaged skin resulting from full-thickness skin wounds, ultimately facilitating the repair of damaged and diseased skin tissue in patients.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Wound healing is a process of paramount importance in preventing infection and restoring the barrier function of the skin following injury. Wounds in complex patients and those poorly managed can lead to developing a chronic wound. This subsequently imparts a

significant burden on the health of human populations, both from an economic and social viewpoint. For example, on average an estimated 3.8 million patients with a wound is managed by the NHS. The cost to the NHS of caring for patients with chronic skin wounds is about £8.3 billion. Approximately 81% of the total annual NHS cost was incurred in the community.

Many approaches are used to speed up wound healing or promote skin tissue replacement and regeneration. For severe skin wounds where significant tissue has been lost, split-thickness skin grafts are the 'gold standard' treatment. Alternatively, synthetic or natural material-based dressings may be used to promote healing. In some cases, growth factors may be applied topically – for example, platelet-derived growth factor has clinical approval. While these strategies may augment and speed up wound healing, none has been shown to promote the regeneration of skin tissues.

A number of biomaterial strategies have shown promising outcomes. However, the mechanisms involved in skin wound repair, especially in the context of chronic (diabetic) wounds highly associated with the immune system, are not fully understood. By Identifying the response of biomaterials to immune cells and exploring their immunomodulatory potential, we can potentially improve the repair of chronic wounds, which currently exhibit low rates of successful clinical outcomes.

Therefore, investigating novel materials that can help skin repair themselves will be invaluable for patients and animals suffering from chronic and acute skin wound injuries. These include non-healing skin wounds due to infection and disease like diabetes.

What outputs do you think you will see at the end of this project?

The proposed model being used in these studies will help us to gain new insights in the development of tissue regenerative biomaterials for skin wound healing
Specifically.

We want to understand how biomaterials (materials, cells, substances of combinations thereof) stimulate immune cells (macrophages) and blood vessel cells to change the inflammation response and regenerate skin tissue.

We hope to develop innovative biomaterial structures that can stimulate skin regeneration in the wound in order to provide new and improved treatments for patients who have large non-healing skin wounds owing to other diseases like diabetes.

We will present our findings at scientific and medical conferences and publish these outcomes in peer-reviewed scientific journals.

Finally, any new procedures/methodologies improved welfare settings developed throughout the period of the project license we will aim to publish and share among the scientific community to benefit both human and animal patients.

Who or what will benefit from these outputs, and how?

As with all our research goals the aim is to develop improved therapeutic strategies and products for patients who suffer from chronic non-healing wounds and skin injuries. We envisage that new biomaterials have the potential to provide improved treatments to help modulate inflammation and rebuild skin tissue within the timeline of the PPL. In addition,

the findings from wound healing research can be applied to the longer term veterinary field due to similarities in dermatological conditions between people and animals, leading to novel applications in a ‘one health’ approach.

Long-term beneficiaries will include:

- Patients and animals suffering from chronic non-healing wounds and skin injuries.
- Healthcare providers.
- UK, EU and worldwide tissue engineering/biotechnology companies involved in tissue regeneration, stem cell biology or developing innovative tissue scaffold technologies.
- The academic community in the generation of new protocols and avenues for tissue regenerative research.

Many materials, cells, substances of combinations thereof we use in our studies are biocompatible and currently used in clinical practice for other applications. Modifying them to enhance their properties can result in potential new therapies in a relatively short time frame. However, with the materials, cells, substances of combinations thereof proposed the final outcomes for patients may require months to years of investigation due to thorough testing to ensure safety and efficacy prior to use in the clinic.

When these new treatments become available, we envisage growing the tissue constructs in the laboratory and transferring the regenerated samples to the patient in theatre. Ultimately, we believe this work will be translated to the clinic and benefit patients within the National Health Service and the wider medical community in the longer term area of wound repair. Alternatively, the development of materials that exploit the regenerative potential of the patient's own repairing cells would minimise longer term costs and hasten the material's therapeutic implementation.

In addition, the information from this project, including the study protocols and techniques will be made freely available within the timeline of the project and in the longer term via publication in peer-reviewed journals, in order to benefit patients, other researchers, doctors, vets, and pharmaceutical companies who are involved in the development and assessment of novel therapeutics which target the repair and regeneration of skin tissues due to disease and injury.

How will you look to maximise the outputs of this work?

Collaborations

This project involves the application of multidisciplinary skills and knowledge, which will be executed through an extensive network of collaborations within our establishment as well as established national and international partnerships. Through these collaborations, we aim to leverage diverse expertise in fields such as skin tissue repair, stem cell biology, immunology, biomaterial development, and real-time imaging techniques like 3D optical imaging.

Education & Public engagement

The information and findings generated from this project will be presented to the scientific community through presentations at national and international scientific research and medical research meetings.

In addition, we are committed to fostering public engagement by organizing outreach activities aimed at GCSE and A-level students. Furthermore, the study's progress and results will be regularly communicated through ongoing teaching events and public lectures.

Species and numbers of animals expected to be used

- Mice: 216

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.
Explain why you are using these types of animals and your choice of life stages.

Whilst all attempts are made to reduce the use of animals by using cell-based laboratory methods, it is inevitable in work of this nature due to a limitation of reproducing angiogenic conditions in 3D structures. Therefore animal investigations have to be undertaken. We can determine the number of factors to ensure the biomaterials (materials, cells, substances of combinations thereof) we make are suitable for wound closure with a number of laboratory methods. However, there are also a number of biological factors that we are still unable to create in the lab that only a living organism can provide.

Typically, a small animal model like mouse is widely used to evaluate the potential of biomaterials for tissue regeneration, because of the low variations among animals, ease of handling and availability, cost efficiency and also because it is easier to compare results between a wealth of experiments reported in the literature. We will therefore use mice in these studies as this model provides the best biological and physiological environment to test the potential of our repair materials to regenerate a significant amount of skin tissue. In particular, we will use wild-type and diabetic (db/db) adult mice to evaluate and compare the efficacy of biomaterials (materials, cells, substances, of combinations thereof) in healthy and diseased conditions.

Typically, what will be done to an animal used in your project?

Through a surgical procedure mice will have full-thickness skin wounds created on their backs. Biomaterials will be administered to the wound site and covered with a dressing. Negative and positive controls will be included in the same animal (a maximum of 4 wounds/mouse) to compare and evaluate the wound regenerative potential of our biomaterial. Pain relief will be administrated pre- and postoperative analgesia as well as during dressing changes. During dressing changes, to minimise distress to the animal we will provide inhalation anaesthesia.

At the end of the study, animals will be killed and when dead the implanted skin area will be removed and fixed for carrying out histology and immunohistochemistry to assess newly generated tissue and cell types.

What are the expected impacts and/or adverse effects for the animals during your project?

This project involves making wounds in the back of mice that are not bigger than 8mm in diameter. In this model, the overall severity the animal will experience is moderate short-term pain due to the procedure. We will give painkillers to mice the day before, during, and 24 hours after surgery. The mice are given general anaesthesia during surgery.

Mice generally tolerate skin wounding well, and in wild type mice, approximately 30-50% wounds are healed within 7 days (wound closure in db/db mice is slower than wild type). There is a small scar, but otherwise the animal remains healthy. We do not expect to see anything more than small changes in the condition or the behaviour of the animal - if more obvious changes in clinical condition (for example, hunching, lack of grooming, hairs standing on end, or behaviour indicating distress such as subdued and self-isolation) occur then the animal will be killed humanely. We also separate the mice after surgery while a dressing is in place, so that they cannot hurt each other or affect each other's wounds. When we do this, we also provide enrichment in cages - this consists of cage furniture (a mouse house) and extra bedding. We will aim to group house the mice with wounds, however in some instance we may have to separate them to maintain the dressing either individual or groups of 2. Once the wounds are starting to heal we will aim to group house them again.

For around 10 days post-surgery, the wound dressings may have to be changed and treatments added. We do this under inhaled general anaesthesia, similar to 'gas' that humans are given at the dentist. This makes the animals less distressed, and so they do not experience discomfort during this procedure. We expect no ill effects from these procedures, though we will humanely kill immediately any suffering or distressed animal humanely.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

For the skin wound defect models in this protocol, we estimate the severity to be moderate and that 100% of the animal will experience this due to the surgical procedure.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Wound healing is a complex physiological process involving a variety of biological systems and cell types. For example, the systemic immune system is involved in regulating healing, and often immune cells are recruited from remote anatomical locations to take part in the

wound healing process. As it is not possible to test the therapeutic efficacy of biomaterials (materials, cells, substances of combinations thereof) in humans at this stage because of ethical and regulatory concerns, we have chosen to use mice as our experimental organism. Despite this, we will replace animals in our research by conducting extensive in vitro experiments to evaluate cell responses on the biomaterials. Where possible we will utilise our organotypic/organ culture ex vivo models as a replacement to the animal models to examine the ability of our regenerative strategies to repair wound defects. Finally, to address the efficacy of biomaterials in the repair of skin wounds there requires an understanding of its effects on integration, inflammation and blood vessel formation, that can only be adequately examined in the environment of the whole animal.

Which non-animal alternatives did you consider for use in this project?

Using laboratory cell culture experiments we are gaining as much information as possible to understand the functions and toxicities of new biomaterials being developed. We have organotypic skin models in vitro models that we can test our biomaterials one.

In other studies conducted in our group using biomaterials, cells and organ culture, modelling is being used to predict the ability of the biomaterials to integrate and develop skin in vitro. Once enough data has been accumulated for modelling, this can, in principle, be applied to the animal models used in these protocols when researching new biomaterials. With continual learning of the models and how new therapies interact in repairing and rebuilding new tissue in vitro modelling predictive parameters developed using these machine learning, will, in the future, be one avenue to replace the use of animal models.

Why were they not suitable?

The above strategies are suitable and we do use them to screen, in part, the many biomaterials that are developed. We only resort to animal studies due to the complex factors that are involved in regeneration and repairing of skin tissue. The generation of new tissue requires multiple steps and the interplay of many different dynamic cells, molecules and matrices during the process of tissue regeneration such as inflammation, proliferation, and remodelling.

This interaction cannot be consistently or correctly reproduced under in vitro tissue culture conditions to meet the criteria expected to inform a clinical translation. In addition, in vitro model systems make it difficult to detect unexpected toxicities and observe the integration and degradation of materials over time.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have used our current and past experimental data to inform us of an estimation of the number of animals required per group and the number of control and test groups to give statistically valid experiments.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In the design phase we have used cell culture, organ culture to reduce intra-group variability of biomaterials. This screening has reduced approximately 60% the regenerative biomaterials being taken forward for testing in animal studies.
Using the NC3R's Experimental Design Assistant we have calculated the minimum number of animals required for a determined amount of new tissue formation in the test implant material comparison to control (no implants) or control (implanted biomaterials without stimulatory factors). From previous studies, we will be using a maximum of 4 skin wounds per experimental group (i.e. n=4, 4 skin wounds/mouse) to attain significance between the test groups.

In certain cases, mathematical modelling may be used to predict the release of growth factors from biomaterials under certain biological conditions, allowing us to determine how many animals we can exclude while still achieving a meaningful outcome in the experiments. We will sacrifice the animal, extracted the skin and measured healing rate by histomorphometry. This multiple scanning and imaging at different time points reduce the number of animals required in these types of studies.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Multiple analyses will be undertaken on biomaterial samples to choose optimal experiment groups for in vivo work. We will harvest tissues (mouse limbs and lungs) from other groups and isolate cells such as macrophages, stem cells, and endothelial cells. We will investigate the cell responses to the biomaterials by biochemical and molecular analysis and also how they respond when cultured on 3D skin The multiple data will lead to an optimisation of experiment groups and a reduction in animal use in a single study.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice have been widely used to assess the benefits of biomaterials to regenerate tissue. The skin wound defect model causes the least pain and suffering; pain responses increase within 2 hours postwounding and analgesia decreases the pain response at 1 and 4 hours. We use the grimace scale to assess levels of pain in the mice. We will therefore provide pre- and post-operative analgesia as well as during dressing changes (inhalation anaesthesia).

Why can't you use animals that are less sentient?

In vivo models are the most predictive model for studying wound healing, allowing a realistic representation of the wound environment including various cell types, environmental cues and paracrine interactions. The most widely used species are mice due to easy handling, housing, and maintenance, and the wide variety of specific reagents for research purposes.

Unfortunately, we are still not at a stage to incorporate all complex biological environments of inflamed wounds in the laboratory therefore these animal models are required to help us understand and improve the development of materials to aid in skin tissue repair. We can mimic some of the components in the laboratory, and this has led us to refine the materials and cells that we will be using in these studies.
The organotypic skin culture model is useful to determine the efficacy of the biomaterials in biocompatibility and vessel formation. This model allows for the assessment and function of these biomaterials in a 3D tissue. However, these are short-term models and cannot mimic inflamed wound defect environments.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We shall continue working to minimize welfare costs for the animals in the following ways:

- Reviewing the need for these experiments to be undertaken. Weighing up the balance of potential successful outcome of undertaking the experiment and the level of harm/distress the animal will endure.
- We will ask for feedback from technicians and welfare officers and their valuable day-to-day knowledge to revise protocols to improve the animals' experience.
- Prior to the surgical procedures, researchers will familiarise and handle the animals on a regular basis to reduce stress for both the animal and the user.
- Up to a maximum of 4 wound defects created in the back skin are well tolerated by the animal. This allows for 4 implants to be tested in the animal. This refinement has reduced the number of mice required to carry out a study.
- General best practise guidance for injection and aseptic techniques will be followed. This has been developed and refined as best practise over a number of studies carried out by the group.
- Animals are closely monitored for several days after the skin wound surgery. Pain relief will be administered if signs of pain persist in the animal. Wounds will be carefully monitored to ensure that the dressing has not loosened or come off or that there are any signs of infection.
- The mice will receive analgesia pre- and post-operation at optimal time points.
- During dressing changes, inhalation anaesthesia will be used to minimise distress to the animal.
- There are clinical score sheets to guide us when monitoring the animals. These cover the mobility, food intake and water consumption, fever, demeanour and respiratory rate and are routinely used for all animals for a minimum of 3 days post-operation. We use the grimace scale to assess levels of pain in the mice.
- Delivery of any material to a defect site will be closely monitored to ensure that there are no significant amounts of inflammation above what is expected with normal skin wound repair. If inflammation persists the mice will be carefully monitored and graded using a locomotion scoring system after consultation with the NVS. Any severe soft

tissue inflammation will be treated with antibiotics, and local pain relief will be provided to the animal.
- Animal weight is frequently monitored for the duration of the studies.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the ARRIVE guidelines which provide a 20-part checklist of the minimum information required to be reported by groups using animals in research. ARRIVE guidelines are essential to help
overcome issues in science such as reproducibility, reducing bias and the correct use of statistical methods of analysis.

In addition, we will follow and consult the Norecopa (Norway's National Consensus Platform for the advancement of "the 3 Rs" (Replacement, Reduction, Refinement)) in connection with animal experiments database platform and PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines for better science experiments using animals to ensure that we are using the best-defined models for our work in the investigations of biomaterials (materials, cells, substances of combinations thereof) for tissue engineering and regenerative medicine.

All surgery will be carried out aseptically in dedicated surgical facilities, according to the LASA guiding principles for preparing for and undertaking aseptic surgery.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will consult the NC3Rs website to identify any changes that will be relevant to the workings of this model. In addition, we will follow the latest findings from the Laboratory Animal Science Association (LASA) and the PREPARE guidelines from Norecopa in better planning for research involving animals to prepare for better science and advance the 3Rs. Any changes will be implemented directly through the experimental design, and if necessary, through a project licence amendment.

In addition, we follow the latest publications on using these models and identify any new methods that reduce, replace or refine the skin implant. If applicable to this model in improving the 3R's we will request amendments from the Home Office to adjust the techniques/methods required and training or notification of relevant staff in updated techniques.

Continual training will be undertaken to ensure that users will be proficient in running these studies particularly on the aftercare post-surgery.

Attendance at Animal user meetings and/or discussion at group lab meetings will be undertaken by the researchers involved with this project.

87. Protection against genotoxic metabolites

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, cancer, ageing, neurodegeneration, DNA repair

Animal types	Life stages
Mice	adult, pregnant, embryo, neonate, juvenile, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Identification of genotoxic metabolites and their origins and understanding how they damage different tissues to cause cancer and drive the ageing 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?

A fundamental question which is also pertinent to human health is what factors drive the ageing process and the emergence of cancers. Our previous research has identified a class of reactive chemicals that are produced in our bodies which can damage our DNA,

the ‘blueprint of life’. These chemicals are known as aldehydes, and we have investigated the two simplest forms – acetaldehyde, which is produced when we break down alcohol in our cells and formaldehyde, which we believe comes from many metabolic processes. We are protected to some extent from these chemicals by mechanisms that remove them and by specific repair pathways that fix the damage they cause to DNA. We want to better understand how these protective mechanisms work and identify new naturally occurring metabolites which may be harmful to our DNA. Finally, we will investigate how DNA damage causes brain, blood, liver, kidney, and other vital organs to dysfunction and fail. These degenerative changes may drive premature ageing of damaged cells and turn them cancerous, processes that we wish to better understand.

Identifying metabolic sources of genotoxic metabolites and a mechanistic understanding of two-tier protection may allow the development of new interventions to benefit human health. Reducing the genotoxic burden might modify the course of disease in diseases such as Cockayne Syndrome, Fanconi Anaemia, or Aldehyde Degradation Deficiency Syndrome, as well as reduce the incidence of cancer onset or tissue ageing in the wider population. Aldehyde genotoxicity might be harnessed therapeutically as a new approach to treat cancer. Lastly, if aldehydes impact on immune function, this knowledge could help to increase efficacy of vaccines or antimicrobial treatments.

What outputs do you think you will see at the end of this project?

Our research using specific and bespoke animal models will provide fundamental insights into the origins of harmful metabolites, from dietary sources to metabolic pathways. Understanding how different cells in the body clear these toxins and repair the damage they cause will help us to develop preventive strategies to limit our exposure and mitigate the toxic effects of these chemicals. This knowledge will contribute to explaining, e.g. how alcohol, a potent source of toxic aldehydes, damages the developing embryo, damages vital organs, such as the liver, and causes cancer.

In summary we expect to:

Explain precisely how alcohol damages our cells and causes cancer; Identify dietary sources of toxic metabolites;

Identify degenerative processes that drive ageing of different organs and give rise to cancers; We will publish our discoveries in prestigious and open access journals;

We will generate new mouse models of human disease (including premature ageing and cancer).

Who or what will benefit from these outputs, and how?

Research on alcohol damage (short – medium term): Our research is likely to explain how ethanol, which is widely consumed throughout the world, damages our DNA, and causes cancer. By disseminating our research through lectures, public events, and written communications we hope to improve the public health messaging on this.

Identifying the character and sources of genotoxic metabolites (medium - long term): This may enable the avoidance of certain dietary sources that lead to the production of these toxic molecules. This may be relevant to individuals with three rare but terminal genetic illnesses (Fanconi anaemia, Cockayne Syndrome and Aldehyde Degradation Deficiency syndrome) as well as to people with other medical conditions.

Identifying how DNA damage drives ageing and cancer (medium-long term): this could help to prevent and treat common illnesses that are associated with old age which at the moment pose a significant burden on healthcare systems.

Over the last decade our research has been published in prestigious peer-reviewed journals (short – long term). As such, our discoveries have and will continue to encourage others to research this area which has broad implications for human health.

Our animal models may prove useful for industry to screen agents that mitigate against toxic metabolites and hence improve the ageing process. They might also be of use to other scientists in the field.

How will you look to maximise the outputs of this work?

We already extensively collaborate with local (UK) and international colleagues. Our published data is accessible for anyone to use and, likewise, we will always share our published reagents and animal models.

Species and numbers of animals expected to be used

- Mice: 50,000

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 the most suitable species to reach our scientific objectives because:

Mouse organ systems, being mammalian, closely resemble that of humans, allowing us to study how toxic metabolites and lacking DNA repair contribute to the development of human conditions such as Cockayne syndrome, Fanconi anaemia, etc. In fact, our mouse models resemble these human conditions better than any other model and have already revealed some of the fundamental processes driving the pathogenesis of these disorders.

Mice are currently the least sentient and lowest mammalian species in which protective mechanisms against endogenous genotoxins are functional and their impact on different organs can be assessed in a reproducible way.

Several refined mouse strains and genetic tools which are instrumental for this project already exist.

Life stages (all): Processes we wish to understand might affect animals at every life stage, e.g. exposure to alcohol causes cancer in adult and aged mice, but also affects developing embryos in pregnant females. Additionally, much of our work is focused on ageing and complex organ systems such as blood and immune system, skin, and brain, and metabolic organs such as the liver. Some of these systems might be affected already at birth, other organs, however, will decline progressively and the full extent of damage will only be revealed in adult or aged mice.

Typically, what will be done to an animal used in your project?

Genetically altered mice will be bred, a process that will not in itself cause any harm. Resulting offspring might have new genetic traits which may change e.g. the way mouse cells respond to DNA damage. Alternatively, we can switch on or off different genes in animals by giving them certain substances (e.g. Tamoxifen) or injecting them with viruses carrying DNA-modifying elements (e.g.

CRISPR/Cas9). These approaches will allow us to make new mouse models without the need for extensive breeding.

Mice may be given genotoxins (e.g. by injection or in drinking water), which are substances that can damage DNA. For example, mice can be injected with chemotherapeutics (used to treat cancer patients) or given alcohol or high-fat diet.

Some experimental mice will be aged so that we can study the effects of DNA damage on processes characteristic of old age, such as decline in brain or liver function, or cancer. To measure these effects, we might perform a series of non-harmful behavioural tests or take a blood sample for analysis.

Mice may be conditioned with radiation and then injected with bone marrow stem cells to rebuild their blood system, allowing us to test the potency of the stem cells according to the genetic changes they carry.

Mice with genetic changes to their skin may be shaved or waxed, allowing us to measure the time it takes for the skin to re-grow hair or re-build hair follicles. Some mice may be given alcohol and have their skin exposed to UV light (similar to sunlight) to determine whether these work together to promote a certain type of skin cancer.

Because metabolism impacts how much DNA damage and repair may occur in our bodies, some mice may be put on a restricted diet to allow us to test whether this results in less DNA damage and keeps them healthy for longer.

DNA damage may also play a role in the context of our immunity. To study this, mice might be given vaccines or be infected e.g. with flu, so that we can measure their immune responses.

Several substances might be given to mice shortly before they are humanely killed. These substances might for example, label certain cell populations or induce a DNA damage response in the cells. These substances will not cause any harm to the animals but will help us to extract maximum information during the analysis of the samples post mortem.

What are the expected impacts and/or adverse effects for the animals during your project?

Adverse effects on this protocol will be largely related to the genotype of experimental mice, age- related changes and/or exposure to substances, such as chemicals that damage DNA. The most common adverse effects include:

Changes in body mass, including obesity or weight loss (short-term, e.g. a few days, to gradual, e.g. a few months);

Development of internal tumours (animals will be humanely killed at onset);

Development of skin or Zymbal's gland tumours (animals will be humanely killed before tumours reach 1.2cm);

Development of blood disorders including blood cancer (animals will be humanely killed at onset);

Stunted growth and premature ageing leading to shortened lifespan (e.g. 12 months instead of 24);

Decline in brain function over time (manifesting at old age);

Effects of infection, including weight loss (short-term, typically 2-3 weeks).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Based on our experience we expect the following proportions for each of the severity categories: Sub-threshold, 50%

Mild, 30%

Moderate, 20%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The main reason for the use of these animal models is that we intend to investigate how DNA repair pathways enable normal development and help mammals to deal with common toxins present in our environment and diet to preserve stem cells, sustain the development of immunity, maintain organ function, prevent neurodegeneration, and finally to protect against changes in the DNA that lead to cancer. These are often systemic processes that cannot be faithfully recapitulated in vitro and are not possible to unpick using human samples. Furthermore, to study stem cell biology, neurological processes, and cancer in a way that can be compared to the human situation, it is necessary to use animal models that are mammals like humans. For these reasons, the mouse is the best model at our disposal.

Which non-animal alternatives did you consider for use in this project?

We are making use of cell lines, including E14 mouse embryonic stem cells and various tissue-specific cell lines such as TK6 human lymphoblast cells and 32D mouse pre-B cells, for use in assays such as cytotoxicity assays and some mechanistic studies.

Why were they not suitable?

While they are useful for specific applications, cell lines are not able to recapitulate the development and ageing of tissues and organs or complex organ system such as the immune system or brain.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have based the number of mice on our past usage from each relevant protocol, the number of mice required for the types of experiments we are planning to do, along with current considerations we have gathered where the genetics of some of our strains do not follow Mendelian rules.

We have used our previous Home Office returns to assist us in assessing the numbers we require for our breeding programmes. We have also taken into account new technologies that we have developed to obtain mutant mice without the need of extensive breeding. For experimental protocols, we have performed power calculations to ensure that we use the minimal number of mice required to obtain meaningful data; however we see considerable sexual dimorphism in some of our phenotypes which will increase the number of animals

required as we need to study both sexes. Depending on the expected effect size and variation, we estimate that typical group sizes will be between 2-3 for pilot experiments, 5-6 for specific molecular read-outs, and 30 for ageing cohorts.

What steps did you take during the experimental design phase to reduce the number of animals being used in this 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 and to assist in maintaining a suitable colony size to ensure only those animals needed for experiments are produced.

We use pilot experiments and experimental software, such as the NC3R's Experimental Design Assistant, to make sure that we use the minimal number of animals required to answer the scientific question. Wherever possible, experiments will use a block design where the predicted necessary cohort is divided into 2-3 smaller cohorts. This design will ensure reproducibility across individual experiments and can incorporate a pilot experiment if one is performed. Furthermore, cohort sizes can be extended if the required power is not achieved, thereby avoiding under-powered experiments.

Variability will be reduced by the use of inbred strains and age-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?

We have already developed several new tools to obtain mutant mice without the need of extensive breeding and will use them wherever possible. When tissue/cell yields are not limiting, we share materials in the lab and/or multipurpose what is done for a mouse's data and/or samples (e.g. body weights or tissues from the same mouse can be used as a control in different experiments) to maximise the data output obtained from a single 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.

Mutant mice that we expect to develop signs of disease will be identified very early based on their genotype (14 – 21 days old). These mice will be monitored carefully by daily inspection for signs of disease and also through regular weighing. Mice developing signs of disease will be killed and analysed promptly to avoid unnecessary suffering.

We have invested in genetic tools that allow us to control where/when mutations come into effect. This allows us to further refine our models. For instance, DNA repair can be switched off or on in specific tissues and/or at specific times. This allows us to limit DNA damage accumulation to one tissue without causing collateral damage elsewhere in the body, resulting in a milder phenotype than in a conventional, globally deficient animal. In the case of inducible mutant models such as tamoxifen- driven Cre recombinase, this allows us to generate mutant mice when we need them, further reducing the possibility that the mice will develop disease when not in an experiment. Similarly, we will generate new somatic mutants using genome-editing tools delivered in the form of viral vectors which have the potential to induce the desired genetic perturbations at a specific time and in a specific tissue, at any stage of animal life as required. We will also be making use of degron-tagged alleles which allow depletion of a protein of interest in mice following treatment with the appropriate ligand (e.g. auxin/AID or dTag systems). Such systems allow for the generation of mouse models in which two-tier protection can be temporally switched off either globally, or in specific tissues when combined with conditional deletion of untagged alleles. A significant advantage of this is that all mice born will remain wild-type for the tagged protein until treated with the ligand, meaning adult models of two-tier deficiency can be generated without the defects in embryonic development associated with constitutive two-tier knockouts. This represents a refinement in the way in which two-tier deficiency is introduced, which will reduce the severity of the phenotypes observed, and will facilitate a reduction in the amount of breeding required to generate experimental cohorts.

Generation of bone marrow chimeras and adoptive transfer of cells is a complementary approach to study cell-specific function in the haematopoietic and immune systems. Where possible we will avoid conditioning steps, but to ensure reliable engraftment in many situations we need to make space in the bone marrow for the graft cells. The standard in the field to achieve this is using total body irradiation (as is used for human bone marrow transplants), which is accompanied by transient weight loss.

Importantly, the adoptive transfer approach allows us to harvest bone marrow from a disease-prone animal before onset of symptoms, harvest, freeze, and treat bone marrow in vitro before transferring them into a recipient animal. This avoids suffering related to potential disease in the bone marrow donor, and reduce the need for treatment in vivo.

As part of this programme of research, we wish to understand how genotoxic metabolites contribute to diseases that are common in humans and generally manifest over time and in particular with age, such as liver or kidney failure and cancer. To test this experimentally, some animals will be aged since we anticipate that like in humans, ageing will play an important role in disease development.

In some contexts, we will use exogenous administration of genotoxins (e.g. ethanol or methanol) as a further refinement - this allows us fine-tuned control over exposure, doses, and durations, and accelerate the onset of phenotypes to shorten the overall extent of animal suffering. We will generally use the minimum required dose and least invasive route that achieves a reliable effect (e.g. preferring administration in the diet or drinking water over injection). However some substances result in aversion, which would result in dehydration and weight loss, and thus gavage or injection may be a more refined route in those instances as it reduces these side effects.

Why can't you use animals that are less sentient?

Mice are considered the least sentient mammalian species, they show human-like development, a comparable blood system, and analogous developmental pathways. They are the best available model to recapitulate the human systems of protection against genotoxins that we are interested in, and which involve complex interplay between organ systems. For instance, food might contain a precursor substance, which is converted to a genotoxin in the liver, transported through the bloodstream, and excreted through the kidney. To study this, we need mice to reach the free-feeding stage. Many changes of interest take time to develop and are age-dependent, and thus a substantial proportion of our work involves adult and aged mice.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We are constantly looking to refine our procedures. When possible, before administering a new treatment, we conduct pilot experiments to test dosages and choose the most effective dose so that we can maximise the desired effect and thus minimise the number of animals we use. We adapt our monitoring, such as by weighing mice more often or starting monitoring at an earlier age, to catch mice that may be developing a phenotype and need intervention.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use the LASA, ARRIVE and PREPARE guidelines as well as the Guidelines for the Welfare and Use of animals in Cancer Research.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We keep up with the current research in our field by reading new publications, and attending conferences and seminars where new techniques and practices are presented. We will attend internal welfare and 3R's meetings where the most up-to-date information is disseminated. We are also signed up to the NC3R's newsletter and regularly check the website for new initiatives. We will also draw on the experience and enthusiasm of our NC3R regional manager, and will also liaise with the Named Information Officer.

88. Mucosal pathogenicity and host immunity

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Fungi, bacteria, host immunity, mucosal immunity, infection

Animal types	Life stages
Mice	adult, pregnant, juvenile, neonate, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The project will target host and microbial interactions, predominantly at mucosal surfaces, with one fundamental objective: To identify fungal and bacterial factors that promote mucosal/invasive infections and the host immune mechanisms that protect against 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?

Infections of mucosal (mouth, gut, vagina) and skin surfaces are by far the most common infections in humans and are often caused by fungi and bacteria. The impact of these infections on global healthcare and economic expenditures is large and of growing concern. For example, two fungi, Candida and Aspergillus, cause the majority of infections in humans, with vaginal Candida alone causing ~100 million infections/year and oral

Candida causing ~10 million infections/year. In patients with compromised immune systems (e.g. transplant, cancer and intensive care), Candida can cause systemic (blood) infections with ~40% mortality, equating to ~200,000 deaths/year globally. Likewise, Aspergillus causes ~100,000 deaths/year with ~60% mortality. Systemic Candida infections are rising and are now the third most common hospital-acquired bloodstream infection and can be considered as equally deadly as many bacterial septicaemias. Together, fungal and bacterial infections kill millions of individuals each year. Therefore, fungal and bacterial infections carry an immense health burden and represent a major socio-economic challenge for worldwide communities.

What outputs do you think you will see at the end of this project?

Mucosal (mouth, gut, vagina) and skin tissues are of immense importance in host defence and immune surveillance, as it is the initial tissue encountered by the majority of microorganisms. Each tissue site is unique, retaining the ability to maintain health whilst at the same time enabling the host to identify dangerous microbes and to initiate protective host immune defences. In recent years it has become apparent that the mucosal immune system recognises and protects us from the myriad of microbes in ways that are different from the systemic (or whole body) immune system. We aim to determine how the mucosal immune system protects us against fungal and bacterial diseases.

This will lead to an improved understanding of how microbes cause disease and initiate immune responses at mucosal barriers. This new information will be shared through presentation and publication. Any new molecules or pathways evaluated to have therapeutic and/or diagnostic potential may lead to patents, and in the mid-long term, products such as vaccines.

Who or what will benefit from these outputs, and how?

These outputs will benefit the research group and establishment as the project will lead to new discoveries, publications, and grant applications.

Our collaborators and the wider scientific field will benefit from these outputs as new information is made available and our understanding of the topic improves. This will drive new collaborations within the scientific community and with industry as the output from this project in the longer term is translated into clinical diagnostics and therapies.

Crucially, by identifying the mechanisms that advance our understanding of how fungi and bacteria promote disease and how the host immune system protects against disease, we will identify new opportunities to prevent these devastating infections thus having a huge benefit for society.

How will you look to maximise the outputs of this work?

The output from this work will be published in open access journals and widely presented at international conferences. All new models and methods will be published in open

access journals. We will collaborate with international colleagues to answer the most impactful and important questions in the subject area. We regularly engage with industry partners to promote the translation of basic research into output that in the longer term would benefit patients.

Species and numbers of animals expected to be used

- Mice: 7000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The mouse has been selected since its immune system is similar to that of humans and the knowledge gained can be directly transferred. Genetically modified mice may also be used and these will allow detailed analysis of individual components of the immune response. The mouse has provided a wealth of information of direct relevance to many human diseases and it is envisaged that this will apply to the diseases studied in this project. We are using adult mice as the most studied and well known model for infectious disease.

Typically, what will be done to an animal used in your project?

Typically, animals will experience mild, transient pain and no lasting harm from the administration of infectious agents and/or substances using standard routes (intravenous, subcutaneous, intraperitoneal) and protocol specific routes (intravaginal and sublingual). Occasionally, animals will be briefly anaesthetised for procedures causing no lasting harm and only transient distress from the administration of the anaesthetic. Experiments will usually last for 1 to 2 weeks. Vaccination experiments may last for 12 weeks but with infection challenge only taking place for the final 1-4 weeks.

What are the expected impacts and/or adverse effects for the animals during your project?

Six protocols are classified as moderate. For oropharyngeal, gut, vaginal and skin infection models either no symptomatic effects are evident or some symptomatic effects are evident locally (redness, swelling). Colitis infection models may lead to some symptomatic effects or signs of discomfort (abdominal pain, loose stools, rectal bleeding). Disseminated infection models may be ultimately fatal to the animals. However, we will not use death as an experimental endpoint and these animals will be carefully and regularly monitored for distress and symptomatic effect. All animals 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)?

Mice - 20% mild and 80% 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?

A significant proportion of the studies to assess fungal and bacterial virulence and immune responses will be performed using non-animal models such as cell culture lines, tissue-derived cells and reconstituted human tissue. However, these studies although providing valuable information are unsuitable for fully assessing microbial virulence in the context of host innate and adaptive immune responses. Only whole animal models of infection create a realistic indication of the progress of infection in the presence of host immune responses. Whole animal infection models are also required to evaluate the efficacy of immunisation/vaccination regimes, monoclonal antibodies and antimicrobial agents, as only in whole animals can the protective nature of these molecules/agents be properly modelled. However, any potential antimicrobial and/or therapeutic agent must have demonstrated activity in vitro before it will be considered for testing in vivo.

Which non-animal alternatives did you consider for use in this project?

We, and others, have extensively used in vitro models of epithelial, fibroblast and immune cells, and ex vivo models and primary human tissue samples of stratified epithelia and immune cells to investigate and model microbial virulence and host immune responses. Additionally, where appropriate we utilise 3D tissue systems grown with specialised culture plates and medium that permit the modelling of microbial virulence and host immune response in layers of stratified cultured primary epithelial tissue. We are increasingly using genomic techniques and datasets to determine the incidence and relevance of genes and molecules allowing refined targeting of our investigations. We and our local and international network of collaborators are constantly developing new models and tools that can be used as non-animal alternatives. We test and implement these new tools wherever possible in our own research. We also routinely search for new tools through the NC3R website and seminars/conferences, and through publications.

Why were they not suitable?

Whilst in vitro, human ex vivo, 3D tissue models and genomic datasets provide valuable information, they are unsuitable for fully assessing microbial virulence and host immune response as these models do not reflect the entire host setting in microbial disease. Whole animal models permit the thorough and human-representative modelling of complex anti-microbial immunity with all aspects of the immune response present and able to interact/function. Additionally, animal models are required to assess the role of immunotherapeutics, vaccines and conventional therapeutics, as only in whole animals can the effect of these molecules/agents be comprehensively investigated.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 we may use has been estimated based on our previous usage for completed projects and our understanding of what may be required to achieve the scientific aims set out in this project license. Additionally, we have carefully considered our available staff and funding resources and used this to inform our research plan including the number of projects, experiments and mice that may be required.

Experiments will have one primary scientific objective unless more information can be achieved by investigating more than one objective within the same experimental groups thereby reducing the number of mice used.

Typically, genetically altered mice will be compared to appropriate wild type controls with one infection dose, thereby resulting in experimental groups and one control group. Occasionally, agents may be administered and/or different doses or frequencies or timepoints of infection may be investigated. In this scenario, more groups are required, typically up to 6 groups. Additionally, where appropriate, control animals will be shared between multiple experimental groups thereby reducing the overall number of mice used. For all experiments, all relevant tissues will be used and/or stored for analysis and future study reducing the need to repeat experiments.

Group size will initially be determined through resource equation-determined pilot studies investigating the primary outcome needed to statistically achieve the scientific aim. Additionally, previous work from ourselves and collaborators (where appropriate) will be used to determine appropriate group size as to achieve the scientific aim with the minimal use of animals. Typically, calculations suggest we will need group sizes between 6-10 to achieve our scientific aims.

We anticipate the majority of experiments in the project will comprise of one control group and then one or more groups of experimental animals. Experiments assessing the role of a gene will typically use an appropriate control genotype and an experimental genotype. Therefore, our calculations suggest this would likely use 6-10 animals in each of the two groups. Experiments assessing the efficacy of vaccines will typically use an adjuvant control group and then experimental groups comprising different vaccine formulations. Therefore, our calculations suggest this would likely use 6- 10 animals in each of the control and experimental groups. The primary readout for these experimental protocols is fungal burden, and our statistical power analysis is based on identifying a large effect in this readout.

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. We have carefully designed each protocol to maximise the research output using the minimal numbers of animals to achieve our scientific aim. As data is generated, will continue to use the NC3Rs' experimental design guidance and experimental design assistant (EDA) and updated statistical calculations to ensure the minimum numbers of animals are being used to achieve the scientific aims.

Animals will be placed into groups using randomisation tools and researchers undertaking the protocol steps will be blinded to the identification of mice and treatment/protocol groups. Where possible, experiments will be undertaken in both sexes with sex applied as a block in experimental design and analysis.

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 monitored and optimised to ensure the most efficient use of animals. Pilot studies will be undertaken where limited/no preliminary data exists to ensure robust scientific statistical conclusions can be drawn from future experiments.

At the end of the experiment, we will harvest as many tissues as possible at postmortem. 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.

Mice will be used to model mild and moderate severity microbial infections at the oral, gut, skin and vaginal mucosal barriers or in disseminated infection. These models are well established and their initiation is typically through routes that cause no lasting harm and only transient discomfort. For the oral, gut, skin and vaginal models: mice are briefly restrained and/or briefly anaesthetised and microbes are introduced into the mucosal barrier setting usually through placing of a swab or pipetting. For disseminated infection models: mice are briefly restrained and/or briefly anaesthetised and microbes introduced into the blood via a tail vein injection or lung through intranasal/intratracheal pipetting. Typically, only the model of oropharyngeal infection requires anaesthesia and this will be kept at the minimum duration possible for the scientific objective to be achieved. The duration of infection will usually be short (less than two weeks) as scientific readouts in these early time points have proven reliable and valuable indicators of longer-term disease progression. Minimum dosing of infectious agents and modulators of the immune system and/or microbiome and/or genes will be used as to achieve the primary scientific objective with the least suffering and distress. Any potential therapeutic agents such as vaccines will be examined in dose escalation studies to ensure suitable tolerance before being used in infection studies. In all protocols, animals will be carefully monitored for a range of symptoms associated with distress. If any distress is observed, animals will undergo an enhanced daily monitoring program. If distress levels reach predefined endpoints, animals will be humanely killed.

Why can’t you use animals that are less sentient?

Non-mammalian animals are limited in their use because they do not have immune systems that model human disease. Whilst zebrafish models of fungal infection are proving useful for studying pathogen virulence in survival models, no zebrafish model currently exists that appropriately models human infections at the mucosa and permits the investigation of host-pathogen/microbiome interactions at the mucosal barrier. We require species that possess the niche-specific microbiome and accompanying mucosal immunology and are therefore suitable for the modelling of mucosal infections. This project will investigate the immunological role of specific host genes and whilst zebrafish tools are now in place to model the developmental effect of gene knockouts, their ability to model gene knockouts in infectious disease models is very limited. Additionally, whilst zebrafish are gnathostomes and possess an adaptive immune system, the function and capability of this system is not well known and may not reflect vaccine efficacy in humans. Therefore, vaccines and host immune-therapeutics against target genes/pathways will be investigated using mice.

We cannot use embryos or young animals as their immune system is immature and does not respond to infectious challenge in a suitable way to model human disease.

Animals will undergo procedures that result in only transient discomfort and no lasting harm and so anaesthetic in this scenario would only increase distress. We cannot undertake infection challenge experiments using anesthetised animals as these experiments require days/weeks of infection and resulting immunology to achieve the scientific objectives.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All animals that have undergone a procedure are regularly and carefully monitored, animals that have been infected with a microbial agent are frequently weighed, examined and scored according to appropriate distress symptoms and weight loss. Animals undergoing the induction of colitis are frequently weighed and scored according to colitis model-specific distress symptoms. Enhanced daily monitoring is immediately implemented when animals are experiencing distress. Additionally, if a procedure is known to enhance susceptibility to infection and therefore may increase distress, animals are immediately weighed and examined at least once per day to ensure distress and severity limits are not exceeded. If any animal experiences distress and severity exceeding what is defined in each protocol, the animal is immediately humanely killed. Based on our previous experience with these models, the duration of infection will typically be short (less than two weeks) as scientific readouts in these early timepoints have proven reliable and valuable indicators of disease course. All agents (infectious and modulators of immunity/gene expression/microbiome) will be administered as the lowest possible dose and frequency as to achieve the primary scientific objective. Any potential therapeutic will be administered in dose escalation studies to ensure appropriate tolerance prior to infection studies.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The PREPARE guidelines for planning and conducting experiments and Sage Journal article Refining procedures for the administration of substances will be followed to ensure research is conducted in the most refined way.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We are signed up to the NC3R's newsletter and attend NC3R's local seminars and presentations at conferences. We stay up to date with the NC3R's website and implement new 3R's ideas and tools into our research wherever possible. As a group we are committed to open discussions with colleagues and collaborators to ensure that we are implementing the 3Rs actively in our research and identifying new ways we can replace, refine and reduce our use of animal models.

89. Interrogating the gut-brain axis in aging 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

Commensal microbiota, Blood-brain barrier, Neuroinflammation, Chronic inflammation, Aging

Animal types	Life stages
Mice	embryo, neonate, adult, pregnant, juvenile, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To identify how diet and the commensal gut microbiota (those microbes that normally live in our gut without causing disease) interact to influence brain function, in healthy aging, in chronic inflammation, and in neurodegenerative diseases such as 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?

We know that the commensal microbes influence brain function, and we suspect that they mediate many of the effects of a healthy diet upon the risk of developing major brain disorders such as Alzheimer's disease . What is far less clear though, is how these effects occur. This project seeks to address this question directly, studying how diet- and microbe-derived molecules can affect the brain and its response to challenge in healthy aging and disease. If we can understand these mechanisms, we will be in a much stronger place to identify and develop novel therapeutic approaches that can promote brain resilience and lessen the risk of dementia.

What outputs do you think you will see at the end of this project?

The immediate benefits of this work will be an increase in scientific knowledge regarding the mechanisms underlying cross-talk between the gut microbes and the brain. Over the mid- to long-term (beyond the immediate project lifetime) this will enable the development of novel agents and approaches to exploit gut microbes in promoting brain resilience in the face of Alzheimer's disease and other dementias. This new knowledge will be provided in the form of scientific publications and presentations at both scientific and lay conferences and events.

Who or what will benefit from these outputs, and how?

This work will provide immediate benefits to our research group and others working in the field, increasingly a strength in the UK biological research base. The information this project will provide will also be of use for the pharmaceutical and nutriceutical industries, as well as clinicians involved in diet and public health.

How will you look to maximise the outputs of this work?

The outputs of this work will primarily be disseminated through several anticipated scientific publications (in open access journals) and through presentation at national and international scientific conferences. Additionally, and as we have done with discoveries arising from previous project licences, we will engage with the popular press to seek coverage of our work at local and national levels.

Species and numbers of animals expected to be used

- Mice: 3500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We need to use animals to study the interactions between the diet, gut microbes and the brain simply because of their complexity. We do not (yet) have either cell culture or

computer-based models that are sufficiently powerful to answer questions about how these systems interact, and thus animal use is unavoidable.

We have chosen to use mice as the experimental species in this project. We understand mouse biology better than probably any other species, allowing us to answer detailed questions about the mechanisms underpinning interactions between diet, commensal microbes and the brain. The majority of mice we will use will be adult animals, although a small proportion may be killed at ages around birth to provide tissue for primary cell culture experiments. Additionally, as one of the principal goals of the project is to understand how diet and the commensal microbes can influence the risk of age-related dementia, we will need to study aged as well as adult mice.

Finally, we will also perform some experiments using genetically altered mice designed to acquire symptoms of Alzheimer's disease as they age. In this case, we will use adult rather than aged animals as our aim will be to delay the onset/lessen the symptoms of Alzheimer's through diet- or microbe- based interventions, and we will be able to detect whether this is working without needing to wait for extensive age-related loss of brain functions that could cause suffering to the mice.

Typically, what will be done to an animal used in your project?

Typically animals used on this project would receive treatment to modulate gut microbe populations over an extended period of time, either by treatment with antibiotics or by feeding altered diets/drinking water. These will be followed in some cases by establishing either acute (abdominal inflammation) or chronic inflammation (gum disease). The effect these treatments have on the brain and its defences will then be assessed using a number of techniques described below. These experiments will be performed on young adult and aged wild-type mice to explore how aging affects this microbe-brain communication and whether microbe-targeting interventions are protective. We will also perform similar experiments on genetically altered animals prone to developing Alzheimer's disease to determine how commensal microbes can affect disease development and if they can be targeted to reduce disease severity; all work will be completed on animals at ages prior to overtly detrimental clinical symptom onset.

Having established these models, animals will then be tested for a) brain function measured by monitoring their performance on different behavioural tests, b) changes in brain structure over time, assessed by non-invasive imaging techniques, c) the integrity of brain blood vessel walls monitored by injection of tracer chemicals or d) for their response to neurological damage caused by direct injection of toxins into the brain. Animals may receive a combination of behavioural testing or non-invasive imaging with either tracer or toxin injection, but they will not receive both tracer and toxin treatment.

a) Behavioural testing

Some animals will then be used for behavioural testing, exploring aspects of memory, attention and emotional behaviour over several testing sessions. To test anxiety, animals

will be placed in either open lidded cages or in mazes with one half enclosed and one half open-walled, and their behaviour will be videotaped. Mice will naturally explore new environments, but will tend to stay closer to the cage walls or concealed spaces than in the open; the proportion of time mice spend in each part reflects their anxiety levels. To test memory, mice will be placed in a maze with different visual markers, and their ability to remember these when they are changed will be assessed by measuring how long they spend exploring unfamiliar markers. To test movement function, mice will be tested for their ability to walk across narrow beams (placed a short distance above soft padding) or on a rotating bar (again, above soft padding) and their ability to maintain balance at different rotation speeds will be tested. To test social function, mice will be micro-chipped with a small radio transmitter under the surface of the skin and will be monitored over an extended period in their home cage, remotely monitoring interactions with their cage-mates. Some of these tests may take place in the presence of distracting stimuli such as lights, noise or coloured objects to test the animal's ability to maintain attention.

Different behavioural tests will be spread out in time, and animals will not be tested in more than one stressful test per day.

b) Non-invasive brain imaging

Some animals will be anaesthetised using a general anaesthetic and will be placed in a non-invasive imaging machine of several different types (e.g. an MRI, ultrasound or CT scanner) to analyse different aspects of brain structure. In some cases animals may also be injected with tracer chemicals to allow us to assess brain blood vessel wall integrity or the degree of inflammation in the brain. This may be repeated up to a maximum of 14 times over a four-month period.

c) Brain blood vessel integrity

Some animals may be injected intraperitoneally or intravenously with a tracer chemical that will allow us to monitor brain blood vessel wall integrity. Animals will then be killed a maximum of 24 hours later.

d) Response to neural injury

Some animals may then receive an injection of inflammatory toxins into the brain. In this case, animals will be deeply anaesthetised, injected with prophylactic pain killers, and placed in a frame to hold them still. The skull will be exposed and a small hole (1 mm diameter) will be made using a dental drill. A needle will be lowered through this hole into the brain tissue and an injection of up to 3 microlitres of liquid containing the toxin will be made. The skin will be resealed using surgical sutures and the animal will be allowed to recover in the warm. Mice will be monitored closely for the next 48 hours, receiving at least one further injection of painkiller 24 hours after surgery, and then kept for a maximum of one month before they are killed.

Animals may also be treated with pharmacological agents or microbe-derived chemicals to test for protective effects upon these different parameters. Animals may undergo a combination of assessments, but no animal will receive more than one surgical intervention.

What are the expected impacts and/or adverse effects for the animals during your project?

Most of the procedures on this project are not expected to have significant adverse effects for the animals. Treatment with microbe-derived chemicals through the diet/drinking water or by direct injection (including tracer injection) are not expected to cause anything more than transient mild distress. Deliberate stimulation of acute inflammation is likely to cause temporary sickness-type behaviours, but animals should fully recover within 24 hours. Induction of chronic gum disease is not expected to interfere with feeding or to cause any noticeable suffering. Behavioural tests may prove stressful to the animals, but will not cause any lasting harm beyond the duration of the test (up to a maximum of 30 minutes for all tests but the home cage assessment of social behaviour, which will be entirely observer independent and performed in the animals' normal cage environments). Non-invasive brain imaging will require general anaesthesia, but animals would be expected to recover fully from this in a short time as the level of anaesthesia needed is only mild and body temperature and fluids will be maintained throughout. Some animals may undergo repeated imaging at different times, which may be a source of stress by accumulation, but this repetitive imaging will be kept to a minimum, and will be spaced across different days. The greatest source of adverse effects will be the model in which inflammatory toxins are injected into the brain, which can include pain caused by the minor surgery upon recovery from anaesthesia (although this will be mitigated by analgesic treatment).

Expected severity categories and the 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 procedures will be classified as of mild severity; intra-brain injections of inflammatory toxins is classed as a moderate severity procedure. Animals genetically altered to develop symptoms of dementia are also classed as moderate severity given the extent of neural damage that occurs at later stages in this strain; we will not keep mice until these ages however.

The proportion of mice receiving a mild severity treatment will be approximately 70%, with approximately 10% receiving the moderate severity brain injection procedure and the remaining 20% being classed as moderate severity due to their genetic alteration.

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 investigating how commensal microbes, diet and inflammation can together influence the brain and the risk of developing neurological disease. As such, we will be examining changes in several highly complex interacting systems, namely the immune response, the microbial communities of the body, and the brain. Currently, there are no computer models able to effectively replicate these complexities. Similarly, the immune response and nervous systems of non-protected animals are too simple to model the interactions that occur in mammals, and we would not be able to satisfactorily address our research questions using these organisms. Our general approach is to address our specific research questions as far as possible using in vitro techniques or through analysis of human clinical samples first, before moving to animal experiments only when all other approaches have been exhausted.

Which non-animal alternatives did you consider for use in this project?

Wherever possible we will address our research questions through the use of immortalised cell culture models (primarily of human origin), taking the information we can gather from using these systems as far as possible. This will be supplemented by analysis of pre-existing public data from human and animal research databases, using bioinformatic approaches for both the generation of initial hypotheses and preliminary testing.

Why were they not suitable?

Whilst both of these techniques can provide a significant amount of information, and we have used them widely in the past to further our research, they remain limited in what they can tell about the complex interactions between diet, microbes and the immune and nervous systems in living organisms. We have no alternative to using animals to investigate how these factors interact and how they can be exploited to reduce the risk/potentially treat neurological diseases such as dementia and Alzheimer's.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 the experiments in this project have been carefully designed to use the absolute minimum number of animals, through rigorous statistical analysis of the numbers of animals required to efficiently detect biologically meaningful differences in our experiments, informed by our previous experience in the field and by the scientific literature.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have employed both statistical tests and online tools such as the NC3R's experimental design assistant to aid in designing experiments using the minimum number of mice possible to detect differences that are both statistically significant and biologically meaningful (typically >10%).

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 make use of non-invasive imaging techniques to permit repeated analysis over time of individual animals, significantly reducing the number of animals needed.

Small scale pilot studies will be used to test the likelihood that agents we plan to administer will have detectable effects on the body, limiting unnecessary use of animals.

Before embarking on large scale experiments, it is our standard procedure to investigate which other groups within our university and beyond may be able to make use of tissue arising. We are keen to ensure that as much benefit as possible is made from the animals used in our experiments.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The models and methods we will use in this project are by and large non-invasive, with treatments through diet or by use of genetically altered animals being our principal approach, with analysis either by behavioural assessment or imaging coupled with post mortem study. This is a deliberate choice, aiming to minimise stress and suffering to the animals. Where we will use invasive studies, these will be either of short duration and only

transient impact (e.g. intraperitoneal injection of pro-inflammatory substances or tracer chemicals), are of sub-clinical impact (e.g. models of gum disease) or will be mitigated by use of anaesthetics and pre- and post-operative pain relief (e.g. injection of inflammatory toxins directly into the brain). Our aim throughout is to minimise pain, suffering and distress, both for animal welfare reasons and because these will compromise interpretation of our experimental results.

Why can't you use animals that are less sentient?

The mouse has been exceptionally well characterised in terms of its physiology, genetics and microbial communities, and represents the best available in vivo model system to ensure results are applicable to human health. Moreover, there are a large number of genetically modified mouse strains available, which will allow us to examine the role of specific components of the immune, nervous and vascular systems in mediating communication between commensal microbes and the brain. The use of such strains will significantly enhance the precision of our experiments, ensuring that the data we obtain has greater clarity than can be gained from studies of wild-type animals or those of other species. Less sentient species cannot adequately model the complex interactions between diet, microbes, and the immune, vascular and nervous systems that we are studying and cannot provide us with the information we need.

Many of our experimental questions refer to the long-term effects of diet and/or microbial changes, and the influence these have on aging. As such, these questions cannot be addressed using juvenile animals as aging is itself a fundamental feature in understanding conditions such as Alzheimer's disease.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The majority of procedures to be employed in this project will be non-invasive, and will involve dietary changes combined with behavioural or post mortem tissue assessments. All animal handling will be according to best practice and in a refined manner. Where invasive techniques are to be used, post- operative care is a priority, with analgesic use and frequent monitoring being a key part of the follow up procedure. In this way, we aim to ensure that while some welfare costs are unavoidable as part of the experimental procedures to be followed, these will be reduced to a minimum.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We have employed the NC3Rs experimental design assistant tool to ensure our experiments are efficiently designed in the most refined way. Additionally, all our experiments involving animal use will follow the ARRIVE and PREPARE guidelines to ensure experimental refinement.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We are in close contact with both the NVS and NACWO at our establishment, who both advise on 3Rs techniques. Additionally, we will engage with the NC3Rs and others to attend relevant training programs to ensure best practice is maintained throughout the period of this project

90. Development and adult life challenges impact on health and behaviour

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

Challenge, diet, disease risk, intergeneration

Animal types	Life stages
Mice	adult, pregnant, juvenile, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to identify how challenges related to diet experienced by the parents before or during offspring development, adulthood or ageing, alter phenotype either at the time of challenge, in later life, or in the next generation, and induce disease risks (e.g., cardiac and metabolic changes, behavioural, cognitive or mental ill-health, vision changes). Based on such new knowledge this project aims to develop and test intervention strategies to protect against increased disease risk in this and future 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?

This work is important as it helps us understand the causes behind disease risks variation and how to decrease them in this and future generations.

What outputs do you think you will see at the end of this project?

This project will lead to new discoveries, peer-reviewed publications, public engagement activities. It may also lead to interventions mitigating changes, prior to and during development, that lead to post- natal disease.

Who or what will benefit from these outputs, and how?

The scientific and medical communities will benefit from the new knowledge generated by this project. Moreover, the general population will also benefit through the public engagement activities linked to the project that will raise awareness of pre-natal environmental/parental risks on later development of offspring, and of potential strategies to decrease disease risk in this and the next generation.

How will you look to maximise the outputs of this work?

We will collaborate with relevant colleagues as and when the project requires it. We will disseminate the knowledge generated through in-house seminars, national and international scientific meetings and peer-reviewed publications as well as public engagement activities. We will ensure unsuccessful approaches are included in our outcomes as the knowledge that such an intervention fails to reduce a disease risk is as important than knowing those that function.

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.

Due to ethical and generational time constraints, phenotype alteration and disease risks cannot be measured directly in humans. There is no alternative to the use of animals to directly determine the timeline between challenge exposure, the mammalian developmental process and subsequent adult phenotype all of which are complex multi-organ system interactions and multigenerational analysis. Tractable non-mammalian

species do not include uterine foetal development making them entirely inappropriate. Mice are chosen as appropriate model based on the large body of knowledge available derived from and available through studying rodent reproductive and developmental biology and their short generation span. Mice and man share up to 99% of genes. Due to the complexity of interacting bodily functions, in vitro systems cannot provide a viable alternative for this research.

Typically, what will be done to an animal used in your project?

Typically, animals will be subject to some diet modulation (e.g., increased fat, reduced protein) for a variable number of days or weeks (typically between 3 days and 6 weeks). Before, during or after diet modulation, animals may also be subjected to a step to improve their physical fitness (e.g., voluntary wheel running, typically between 3 days and 6 weeks) alongside the diet modulation. To record body status and physiology parameters at baseline, during and just after the challenge related to diet, animals may undergo body status measurement (e.g., blood pressure measurement). As the objective is to measure the effects of the challenge in this or the next generation, the animals may also be bred before, during or after the challenge.

To study the effect of this challenge, mother and offspring will be analysed in a broad range of optional assays. These will include uterine tract analysis, growth measurement and blood pressure measurement. We will assess body status measurement. Further outcomes that will be assessed will be metabolic profile using glucose tolerance testing, immune system testing, behaviour testing.

Animals may also be subjected to assessment of the visual system, which may require general anaesthesia. Animals will then be culled for tissue collection.

What are the expected impacts and/or adverse effects for the animals during your project?

Most of the changes we expect to be sub-clinical e.g., slower learning responses, or raised blood glucose levels. However, giving multiple doses of anaesthetic may have an intrinsic risk. Blood sampling will be lancing or needle stick and may cause momentary pain.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severity for this proposal is at moderate level, with 80% of animals expected to experience this level. This is a cumulative severity, individual steps are either mild or moderate, with 80% of animals expected to experience at least one step or accumulate steps to reach 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?

The purpose of our work is to analyse how specific challenges impact on development and adult health, including across generations. Due to ethical and generational time constraints, this cannot be examined directly in humans. There is no alternative to the use of animals to determine directly the timeline between challenge exposure, the mammalian developmental process and subsequent adult phenotype. It is essential to use mammalian species to study in vivo conditions integrating all aspects of factors found in living animals and humans.

Due to the complexity of interacting bodily functions, in vitro systems cannot provide a viable alternative for this research aim. In vitro systems will be used to supplement the work when focusing on specific steps (e.g., stem cell differentiation and the effect of interventions with specific compounds), however the information they give will be more limited compared to that obtained from the multi-system physiologically relevant in vivo models. Given this context, we are not aware of any alternative to the use of animals that could be considered.

Which non-animal alternatives did you consider for use in this project?

We will and do use in vitro approaches utilising stem cells. We will and have in the past derived embryonic, epiblast and trophoblast stem cell lines from embryos previously environmentally challenged to allow amplification and bulk analysis of phenotypes. This strategy has proven effective to determine detailed mechanisms. We have already derived some of these lines from different challenge models to reduce the number of animals required. Studying such programmed cell lines would teach us how phenotype and physiology of these early cell lineages have been shaped through the prior environmental treatment and facilitate testing of interventions with specific compounds. However, differentiation of these cell lines is limited and prone to artifact hence such information will still require confirmation in an in vivo setting, but this will reduce the number of animals required through facilitating a more directed experimental design.

Why were they not suitable?

As mentioned above, in vitro approaches complement animal research and will inform the design of animal experiments by facilitating a more targeted experimental design. The in vitro approach doesn’t replicate the complex developmental and intergenerational changes

occurring in vivo. Moreover, our project analyses the effect of changes across different body systems as well as the interaction of these different body systems, for which an in vitro approach is not yet 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?

Given the number of groups (3-6) and the number of animals per group needed to have strong statistical power, and with the different experiment designs we describe here, we have estimated the number of mice per protocol. We plan 3 different experiments for protocols 1 and 2, and 1 experiment for protocol 3, which will be repeated 3 times, reaching 300 animals for protocol 1, 2000 for protocol 2 and 700 for protocol 3.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

To minimise the number of animals used we will ensure to limit the required number of replicates according to power calculations based on our past studies. In most cases, 10 animals and offspring per sex will be required per treatment group to achieve statistical significance where a change of 10% on the mean is observed. The NC3Rs EDA was also used. Collaboration with our in-house statistical experts will be continued for statistics advice and refinement of statistical models when appropriate.

Generally, due to the hierarchical nature of the data and potential confounding variables (e.g., parental origin, body weight, litter size etc.) a random effects regression analysis (SPSS) will be employed.

In our project, the exposures are the different diets, and the outcomes are the different measurements and analysis done: physical fitness performance, body status measurement, blood pressure measurement, growth measurement, glucose tolerance test analysis, immune system test analysis, behaviour tests analysis blood biomarkers analysis, fundoscopy analysis, ERG measurement, OCT measurement, optometry analysis. Integrating several outcomes within respective research topics will help reduce animal numbers used. For example, parental and gamete/early embryo outcomes can be collected from the same animal where possible, and body weight, cardiometabolic and behavioural measures, final organ allometry and RNA/protein expression or epigenetic profiles can be collected from each animal. Further reduction of animal use can be accomplished through examining aspects of cell differentiation and proliferation/death during prenatal development using embryonic, epiblast and trophoblast stem cell lines.

To allow longitudinal studies, some genetically altered mice may be used. These mice may express a gene expression reporter for example to allow less invasive measurements at different time points rather than sacrificing different mice at several time points.

The experimental design that we will employ will allow publication of the results according to the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. This will maximize information published and minimising unnecessary duplication of studies. Animals may be obtained from other project licences with relevant authorities (e.g., for genetically altered mice) if appropriate to reduce 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 will plan as much as possible to combine different analysis (metabolism, behaviour, cognition, visual acuity) in the same animals provided these are not expected to affect each other's. We will also collect as many tissues as possible once the mice are culled so further analysis can be done independently of the physiology and behaviour tests done on the 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.

This project aims to identify mechanisms how environmental challenges experienced prenatally, especially around conception, impact on postnatal disease risk in the offspring. Therefore, it is essential to use mammalian species to study in vivo conditions integrating all aspects of factors found in living animals and humans. Non-mammalian species do not include uterine foetal development making them entirely inappropriate. As a biomedical model with human relevance, Eutherian mammals are essential since birth is relatively premature in marsupials and their embryonic development is sufficiently different both pre- and post-implantation. Mice are chosen as appropriate model based on the large body of knowledge available derived from and available through studying rodent reproductive and developmental biology and their short generation span. Mice and man share up to 99% of genes. Most importantly, analysis of dietary mechanisms involved in 'Developmental Origins of Health and Disease' as well as embryo in vitro culture effects on long-term health has been firmly established in rodent.

The changes we expect to see here, would be considered generally as subclinical.

Why can’t you use animals that are less sentient?

As our project examines parental to offspring communication as a human model, mammalian species are necessary. The mouse is a tractable system whose genetics, epigenetics, biochemistry and physiology are best understood and can be used to study changes in the adult and aged animal.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Research procedures relevant to the project will not exceed MODERATE severity. To minimize suffering, animals will be assessed for ill health and distress daily (e.g., abnormal appearance, body function or behaviour). Severe malnutrition will not be used in this project. If any signs of ill health, distress, pain or injury are detected, respective animals will be killed by Schedule 1 procedure. Only veterinary approved procedures for anaesthesia will be used. Agreed early humane endpoints are described for each step of the protocols. Mice will be caged with appropriate environmental enrichment and only housed singly when clearly needed, for example when using a metabolic cage. Where tube holder-restraint is given the animals will be habituated to the process before the experiment begins.

We may use radio-operated implants to monitor temperature, activity levels, heart rates and blood pressure in some cases to avoid repeated restraint.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The researchers will stay informed about advances in Refinement by following updates though the NC3Rs and Norecopa websites and newsletters, by reading peer-review articles and discussing refinement good practices with colleagues and animal facility staffs and by actively implementing the latest relevant techniques. We will follow best practice guidance from the ARRIVE guidelines to ensure experiments are conducted in the most refined way and reported fully.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The researchers will stay informed about advances in the 3Rs by following updates though the NC3Rs and Norecopa websites and newsletters, by reading peer-review articles on different replacement techniques, by regularly discussing refinement and best practices with colleagues and animal facility staff and by actively implementing the latest relevant techniques.

91. Development of tumour targeted agents for imaging and treatment of cancer

Project duration

4 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, Radionuclide therapy, Molecular imaging, Biodistribution, Drug delivery

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of the project is to design, synthesise and evaluate molecularly-targeted imaging tracers and drugs that contain a radioactive substance (radiopharmaceuticals) that can be used to detect or treat cancer. Some of these agents are designed to fulfil both of these functions, when they are called "Theranostics".

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 major cause of ill health and mortality in the UK, with nearly 400,000 new cases and 170,000 deaths reported annually. While outcomes have improved considerably for some types of cancer, others remain stubbornly resistant to treatment. Nuclear medicine imaging using positron emission tomography (PET) and single photon emission computed tomography (SPECT) is commonly used to detect and monitor cancer. The development of new sensitive, highly cancer-specific imaging tracers would benefit many patients, including those with cancers that are considered to be hard-to- treat. Radionuclide therapy involves coupling therapeutic radionuclides that emit charged atomic particles to a tumour targeting drug, which could be a small molecule, peptide, antibody or nanoparticle. It combines the potent cancer-killing property of ionising radiation with the ability to deliver the treatment systemically, usually via an injection into the bloodstream. Therefore, unlike external beam radiotherapy, it provides a whole body treatment for when cancer has spread from its original site.

What outputs do you think you will see at the end of this project?

This programme of work is expected to result in:

The development of radionuclide-tagged targeted diagnostic and therapeutic agents that show potential for clinical use in the early detection and/or treatment of human cancers.

Understanding how radionuclide therapy activates the systemic anti-tumour immune response.

Understanding how radionuclide-based imaging and therapeutic agents can be engineered to reach target molecules inside cancer cells not just those on the surface of cells as is currently the case.

Understanding the therapeutic effect of combining anticancer radionuclide therapy with external radiation and other anticancer treatments.

Who or what will benefit from these outputs, and how?

This project will benefit:

The cancer research community as it will provide new information about how best to combine radionuclide-containing drugs with other agents. In particular, on-going research regarding the combination of radiopharmaceuticals with immunotherapy will be reported in the next 2 years.

Throughout the project, the drug development community will benefit, as this research will highlight the potential gains of radionuclide therapy.

The protein engineering, biology, and antibody therapeutics community, will benefit from this project as new approaches to targeting intracellular molecules with therapeutic agents are investigated, with initial reports to be published within the first 18 months of the project.

The primary long term potential benefit of this work (beyond the lifetime of the current project) is that a novel imaging agent, therapeutic agent or combination of therapeutic agent plus external radiation may be taken forward for Phase 1 human clinical trials.

How will you look to maximise the outputs of this work?

Outputs of our research are disseminated via the conventional academic routes of presentation at scientific conferences and publication in peer-reviewed journals. In addition we have a track record of collaborating with cancer research and advocacy organisations in Patient and Public Engagement events. We collaborate widely with colleagues from different disciplines including surgical and medical oncology, nuclear medicine, mathematics, physics, chemistry and immunology, which reflects the essentially multidisciplinary nature of this work as well as ensuring maximal academic outputs of the 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 use mice in our research because in vitro experiments cannot simulate uptake of imaging tracers or radionuclide therapeutics into tumours, the length of time that an agent stays in circulation, or the rate or extent of excretion of the agent via the liver and kidney. Therefore, there is currently no substitute for using animal models in the investigation of imaging tracers and radionuclide therapeutics.

The mouse is used as it is the lowest animal whose genetic, biological and behavioural characteristics closely resemble those of humans. Mice are required for our research to provide biodistribution and pharmacokinetic data, to evaluate the optimal amount of imaging tracer or drug to be injected, and for tumour growth inhibition studies in mouse models of cancer.

We use adult mice because the human cancers that we study (e.g. pancreatic, oesophageal and triple negative breast cancers) occur in adulthood.

Typically, what will be done to an animal used in your project?

We will use models of mice that are immunocompromised to reduce the risk of rejection of the tumour cells we administer. As these mice are susceptible to infection, they are housed in bio secure cages. Where we need a fully functional immune system we will use wild type mice.

Animals will undergo induction of tumours by the injection of tumour cells or fragments. Typically, we use one or two sites in the flank of the animal for these injections which are usually done under general anaesthesia. Tumours will be measured up to 4 times per week usually using callipers whilst the animal is fully conscious and manually restrained. For precise measurements of the tumour the animals may undergo an MRI or other type of scan whilst under general anaesthesia. These sessions are usually around 1 hour in duration.

New imaging tracers will be administered during the tumour growth period, and imaging methods such as PET-CT, SPECT-CT or optical imaging will be done under general anaesthesia to visualise the distribution of the tracer and to see how well it detects the tumour. It may be necessary to repeat the imaging session on up to 6 occasions over the following 4 days, to continue to monitor the distribution of the tracers. Each scan is done under general anaesthetic and takes approximately 1 hour.

For studies of new therapeutic agents, once the tumour has reached the required size, the new drug will be given to the animal, usually via an intravenous injection, just as human cancer patients undergo. Occasionally we may use the intraperitoneal or oral gavage route for drug delivery. The new agents are typically given once but, for some agents, repeat dosing on up to 8 occasions may be done. In some cases, the new drug will be administered in combination with external beam radiotherapy that is targeted precisely to the tumour and delivered in a single or in up to 10 sessions, with the mouse under general anaesthesia. This procedure takes approximately 1 hour. In some cases the new drug will be administered together with another anti-cancer agent. Following administration of the new drug, tumour growth is measured using calipers over time to monitor the response to treatment.

For all procedures involving general anaesthesia, animals are monitored closely afterwards, to ensure complete recovery before another procedure is initiated. For all studies, animals are humanely killed before the total tumour volume reaches 1000 mm3. After this, tissues are harvested to be analysed post-mortem.

What are the expected impacts and/or adverse effects for the animals during your project?

The subcutaneous injection of tumour cells or fragments will lead to the formation of tumours. Typically, we use one or two sites in the flank of the animal for these injections. Superficial tumour burden is not expected to impact on the overall welfare of the animal but occasionally the size of the tumour can cause the overlying skin to ulcerate. These animals undergo increased monitoring to ensure that the ulcer does not progress to full skin thickness or become infected. Tumour growth is limited to a maximum of 1000 mm3.

Weight loss of up to 15% may be associated with tumour growth and/or treatment regimens but will be supported in using moist palatable food as a way of keeping the weight loss to a minimum.

The administration of imaging tracers and imaging sessions under general anaesthesia are not expected to cause any harms. Nor do we expect adverse impact from the therapeutic agents we plan to use.

Expected severity categories and the 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?

It is known that the anticancer effect of external (conventional) radiotherapy is partly mediated through stimulation of an anti-tumour immune response. We are studying whether this is also the case for radionuclide therapy. The immune system is highly complex, being made up of many cell types and so it is not possible to fully replicate it in the laboratory. For this reason, for some or our research, it is necessary for us to study the effects of radionuclide therapeutics in animals with a functioning immune system.

It is also important to study other aspects of tumour growth and response to treatment in animals. This is because cancer cell models that are cultured in the laboratory do not adequately reflect the complex environment of the body. In vitro experiments cannot provide us with information regarding the extent of uptake of an imaging tracer or therapeutic agent into a solid tumour, the length of time that the agent stays in circulation, or the extent of clearance of the agent via the liver and kidney. Therefore, there is currently no substitute for using small animal models for our research.

Which non-animal alternatives did you consider for use in this project?

We test all investigational imaging tracers and therapeutic agents initially in in vitro models, to confirm efficient targeting of or cytotoxicity towards cultured cancer cell lines before commencing in vivo experiments. Most of these assays are carried out on cultured cells growing as a monolayer, but we also use multicellular spheroids to more accurately mimic microscopic tumours.

Why were they not suitable?

While useful for initial exploratory studies, monolayer and multicellular spheroid models cannot inform about the pharmacokinetics, or fully simulate intratumoural accumulation and tumour growth suppression following administration of a radiopharmaceutical to an animal. Spheroids measure 2 or 3 mm at most and so cannot reflect the biophysical characteristics of a tumour of clinically meaningful size.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimation of the number of animals is based on a review of our Home Office returns over the last 5 years, and on our experience of experiments conducted over the last 10 years that are of similar design to those described in this PPL (and takes into account that the duration of this PPL is 4 years).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

For most experiments we use immune compromised strains of mice to improve the chance of successful xenograft growth and thereby reduce the need to order surplus animals (except for the immune system studies, where it is necessary to use immune competent mice).

In some cases we implant subcutaneous or mammary fat pad tumours in two sites, so that one tumour can act as the internal control for the other, thus reducing the number of animals used.

For tumour cell lines that are difficult to establish as xenografts from in vitro culture, we grow tumours in a small number of donor animals and inject the homogenised tumour into recipient animals. This increases the chances of successful tumour growth and therefore reduces the number of animals used.

For breast cancer studies, xenografts are grown in the physiologically relevant setting of the mammary fat pad. This increases the likelihood that the tumour will grow successfully, leading to minimisation of animal numbers.

When evaluating the biodistribution of a novel agent, we obtain serial images at selected time points, with each animal acting as its own control. This reduces the animal numbers required for the study.

We incorporate the ARRIVE and PREPARE guidelines into the design of our studies.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

When we wish to use a cancer cell line that we have not used previously in xenograft studies (particularly if there is limited published data for the cell line) we perform a pilot study in a small group of animals to gauge xenograft take rate and growth characteristics before using the cell line in a definitive biodistribution or therapy study.

For experiments involving a combination of a targeted radionuclide agent with external radiation, dosimetry calculations (computer-based modelling) are performed in advance to predict the optimal treatment regimen for in vivo studies.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The mouse is the most commonly used model in the field of cancer research and we have extensive previous experience with the mouse as a model for cancer development.

We use xenograft tumour models, in which cancer cells are implanted subcutaneously, subsequently growing into a tumour. In most cases, we use strains of mice that are bred to have an impaired immune system, which means it is more likely that tumours will form.

The subcutaneous implantation of cancer cells results in growth of a tumour (xenograft). This provides a suitable model for testing new imaging tracers that, after intravenous injection, are intended to track to a tumour but not normal organs. We can visualise whether this has happened using methods such as whole body PET-CT or SPECT-CT. If the imaging tracer looks promising it is possible to convert it into a therapeutic agent by attaching a cancer cell-killing (radionuclide) payload. The subcutaneous tumour model is then used as a means of testing the efficacy of the therapeutic.

There is extensive world-wide experience of using xenograft models of human cancer in mice over several decades, so the potential adverse effects, such as skin ulceration or interference with mobility, are well known and so can almost always be avoided.

We limit the allowable size of the tumour(s) to 1000 mm3. Tumours of sizes up to this limit rarely cause suffering to the animal.

Why can’t you use animals that are less sentient?

The imaging tracers and anticancer agents that we are developing are intended for use in human adults. Important aspects to be evaluated are how the agents distribute in normal organs and tumours, the temporal kinetics of this distribution, how the agent is excreted and how efficient it is at eradicating cancer. Only a live mammal can fully reflect or predict how these variables would behave in a human.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Because weight loss is the most obvious indication of suffering in rodents, any animal on any protocol which demonstrates greater than 10% weight loss will receive moist palatable food to encourage and facilitate eating. If 15% weight loss is reached, animals will be humanely killed.

All mice will be humanely killed before they reach the age of 12 months, to avoid age-related complications.

We employ humane killing before combined tumour volume reaches 1000 mm3 for flank tumours or 500 mm3 in mammary fat pad tumour sites to avoid any impairment of mobility.

Imaging tracers or drugs will be administered in the smallest volume that can be accurately/safely administered. We may use the injectable or oral route of administration, and will adhere to the stated limits on the number and frequency of doses. Injections may be administered to conscious mice that are competently restrained, or to animals under gaseous anaesthesia.

Where the administration of more than one agent within a short time-frame is required (for example, the administration of imaging tracer and a contrast agent during the course of an imaging session) an intravenous cannula will be used where possible, to reduce the number of times intravenous access is needed.

Oral drug administrations will be made to conscious restrained animals, and formulations will be flavoured where possible to render them palatable to the animals.

Where established anticancer drugs are to be administered in combination with one of our investigational agents, they will be administered in doses and formulations shown to be well tolerated; either from the experience of colleagues or the scientific literature.

Many of the targeted agents that we are developing incorporate a radioisotope. Radioactive isotopes of iodine are known to accumulate in the thyroid gland, which could result in adverse effects. Therefore, when these isotopes are used, we will administer potassium iodide to prevent thyroid uptake of radioactive iodine.

PET, SPECT, MRI, fluorescent or diagnostic ultrasound imaging may be performed to measure the size of tumours, investigate the vascularity of tumours, investigate the biodistribution of agents, quantify the uptake of agents in the tumour, or provide precise anatomical information to guide the delivery of external radiation. Imaging sessions will generally be performed under gaseous anaesthesia, with body temperature maintained by use of a heated blanket and lubricant eye gel applied. For longer imaging sessions (greater than 0.5 hour in duration) animals may be supplemented with subcutaneous fluids to prevent dehydration. Animals will be checked for complete recovery from previous anaesthetic sessions before undergoing further sessions.

In combination therapy studies in which external radiation is delivered to the tumour, radiation dose to regions other than the tumour is minimised either by shielding with lead or the use of a focused micro-irradiator (SARRP).

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We adhere to the ARRIVE guidelines and make use of the Experimental Design Assistant (https://www.nc3rs.org.uk/our-portfolio/experimental-design-assistant-eda). We also refer to "Guidelines for the welfare and use of animals in cancer research" (Workman P. et al., Br J Cancer, 102, 1555–1577, 2010).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

A member of our research group has served on the local AWERB over the last 4 years, and this continues. This has been a useful mechanism for us to learn about approaches to the 3Rs of other research groups and of the AWERB itself, allowing us to implement these approaches in our own work where appropriate.

We attend internal 3R’s events and departmental welfare meetings. We have access to a Named Information Officer when needed.

We work closely with a medical biostatistician who we routinely involve in sample size selection and other aspects of experimental design, enabling us to implement new approaches to Reduction and Refinement without jeopardising the likelihood of obtaining statistically meaningful results.

92. Evaluation of targets and novel therapies for nucleotide repeat expansion diseases

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Nucleotide repeat expansion disorders, Nervous system dysfunction, Mouse models of disease, New therapies

Animal types	Life stages
Mice	adult, juvenile, embryo, neonate, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of the project is to develop mouse models of nucleotide repeat expansion disorders, which will then be used to evaluate the effectiveness of potential therapeutics. This will aid the development of new pharmacological approaches to treat these debilitating conditions.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these

could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Nucleotide repeat expansion disorders (NREDs) encompass a set of over 50 diseases including myotonic dystrophy, Huntington's disease, spinocerebellar ataxia, Friedreich's ataxia and fragile X syndrome. They are frequently inherited and are caused by the expansion of repetitive DNA sequences.

Fragile X syndrome is one of the most common NREDs, affecting approximately 1 in 5000 people whereas others are rarer e.g. Huntington's disease (4-10 in 100,000 affected). Prevalence varies between populations and can be significantly higher in certain groups. NREDs are associated with a broad range of clinical symptoms which can manifest in childhood or later in life. However, the nervous system is nearly always affected which can lead to movement difficulties, cognitive and psychiatric disturbances, and neurodegeneration. Symptoms generally become worse with time and may result in life-limiting disabilities and in some cases early death. Full time care is often required in the later stages of disease, which places a significant burden on healthcare systems and carers.

For the majority of NREDs, no treatments exist to cure or alleviate the symptoms, highlighting the need for new therapies in this area. This project constitutes part of our efforts to develop novel therapeutics for these diseases, with the aim of reducing neurological symptoms, slowing the progression of motor and cognitive decline, and hence improving patients' quality of life. Given that NREDs have a common underlying cause, therapeutics developed for one disorder may also be effective against other NREDs.

What outputs do you think you will see at the end of this project?

This work is expected to provide data on how well potential therapeutic compounds are tolerated and their effectiveness in treating the underlying causes and symptoms of NREDs. These data will be used to identify candidates for further development. Overall, the project is expected to identify one development candidate per year, from which 2-3 would be expected to enter clinical trials within the five-year lifespan of the licence.

In addition, this project encompasses more exploratory work to validate new biological drug targets for NREDs, which could open up new treatment opportunities.

Who or what will benefit from these outputs, and how?

Testing potential therapeutic compounds in disease models of NREDs will help project scientists establish the relationship between drug exposure and efficacy (compound effectiveness), thereby facilitating the selection of compounds for further development. It will also assist with the identification and characterisation of biomarkers, which indicate the

state of disease. Data from studies may also be used for internal stage-gate documents and regulatory documents, which are required to progress therapeutics to clinical trials.

Ultimately, we hope that people living with NREDs will benefit from these outputs. The therapies being developed and tested have the potential to provide real improvement in the quality of life of these patients, with significantly fewer side effects. Carers are also significantly impacted by these diseases and could benefit indirectly.

How will you look to maximise the outputs of this work?

Findings will be made available to other scientists at presentations and internal meetings to further our understanding in this disease area. We will also take as many tissues as possible at the end of studies, which will be made available to other company researchers thereby allowing the maximum amount of data to be generated from each animal used.

Species and numbers of animals expected to be used

- Mice: 9500. This is the maximum number of mice that can be used over the 5-year duration of the licence.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

All the animals used in this project will be mice. Many well characterised mouse models of NREDs already exist and have been thoroughly described in the literature. In general, the anatomy and physiology of the mouse is well understood and provides the best compromise in terms of correlating with human biology whilst exhibiting the lowest level of sentience.

Embryo, neonate, juvenile, pregnant, adult and aged life stages will be used, but most studies will be performed using adult mice. For studies using early onset disease models, compound dosing may begin at the juvenile stage (3-6 weeks), to allow dosing to be completed before mice reach end-stage disease. For studies using late onset disease models, aged mice may be used because we are assessing a slowly progressing neurodegenerative disorder. All life stages apart from aged will be used when breeding mice for studies.

Typically, what will be done to an animal used in your project?

Disease model mice will be dosed with compounds to investigate their effects on the underlying causes and clinical symptoms of NREDs. Compounds will be administered orally or by injection for a period of a few days (for tolerability studies) or several weeks (for studies investigating treatment effectiveness).

Some mice will undergo surgery, for example to implant electrodes to perform neuronal recordings from the brain. These animals can be kept for up to 90 days so that longitudinal data can be collected.

What are the expected impacts and/or adverse effects for the animals during your project?

We will predominantly use two mouse models of disease. One has late onset symptoms that progress slowly and the other has early onset symptoms that progress more rapidly. Wherever possible, the late onset model will be used. Late onset disease model mice do not generally deteriorate to a state where they require special care and have a normal lifespan. However, early onset disease model mice display neurodegenerative symptoms, which impact on welfare, and include tremor, frequent urination, reduced spontaneous movement and loss of body weight and muscle mass. Up to 5% of these animals may die as a result of seizures. Importantly, they will not be kept to an age where they experience end-stage disease. After onset of disease symptoms, they will be given special housing conditions to ensure they have easy access to food and water, as well as extra bedding to absorb excess urine. These animals will be monitored closely on a daily basis, and signs of distress e.g., sudden loss in body weight, piloerection, hunched posture, reduced spontaneous activity will trigger increased health monitoring. If any further deterioration occurs, animals will be killed promptly and humanely.

Animals are expected to experience momentary pain and stress during administration of therapeutic compounds. A small number of animals may experience chronic compound-related adverse effects during tolerability studies, which are likely to involve body weight loss and a deterioration in clinical signs. Any animals exhibiting such signs will be killed promptly and humanely.

Animals will experience some discomfort after surgery, but appropriate pre-operative analgesia and post-operative care should minimise pain and aid recovery. Serious adverse effects are not expected, but if they occur, they are likely to involve body weight loss and deterioration in clinical signs. Any animals exhibiting such signs will be killed promptly and humanely.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

If animals experience the maximum severity permissible under each protocol - the proportion of animals in each category would be:

Mice - 32% mild and 68% moderate

However, we do not expect all animals to experience the maximum severity permissible. For example, if late and early onset mouse models of disease are not kept until they show clinical signs of disease, then they will experience sub-threshold rather than mild and mild rather than 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?

Animal models of NREDs are essential to assess the effects of test compounds on more complex clinical symptoms such as neurological function and motor abnormalities. Testing in an intact mammalian system is necessary to relate in vitro data to key disease read-outs, in order to accurately predict clinical benefit.

In addition, animal models are needed to determine the relationship between compound effectiveness and the levels of compound found in different tissues, particularly the brain. This relationship is driven by compound absorption, distribution throughout the body, metabolism and elimination, and cannot be accurately modelled in vitro. In vivo techniques more closely mimic the clinical situation, in which compounds will eventually be trialled. It is therefore essential that compounds are tested in more clinically relevant models.

Which non-animal alternatives did you consider for use in this project?

Assays in human cell models give a good indication of the ability of compounds to modulate the underlying causes of disease, and are used to triage compounds before they are tested in vivo. Use of patient postmortem brain tissue has also been considered.

Why were they not suitable?

Cell models of NREDs cannot reliably predict in vivo effectiveness of compounds against more complex phenotypes such as neurological and motor dysfunction. Patient postmortem brain is difficult to source and usually only represents late-stage disease. It is also not suitable for investigating neurological and motor dysfunction.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise

numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals used in the project is based on the work required to test one potential therapeutic compound per year in our disease models over the 5 year period of the licence. This includes development of new disease read-outs and biomarkers. It also allows for the investigation of one new biological drug target, and the establishment of associated models and assays.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have access to an internal Preclinical Statistics Centre of Excellence (CoE), which is a team that assists with experimental design, data analysis and reporting of results. We develop integrated statistics plans with the CoE for all large efficacy studies that pre-define animal group sizes and the statistics to be used for data analysis. Work with the CoE has allowed us to reduce the group sizes required for efficacy studies using an early onset disease model. Pilot data showed that the primary read-out becomes more variable with age, so commencing dosing earlier means that less animals are needed (dosing from 4-10, 5-11 and 6-12 weeks of age requires 10, 12 and 15 mice per group respectively). These types of analyses will be regularly reviewed as more data is obtained to determine whether further reductions can be made.

Where possible repeated measurements will be made in the same animal (e.g., recordings of neuronal or muscle activity). Longitudinal studies reduce the total number of animals used and increase statistical power.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will use efficient breeding strategies to generate cohorts of genetically altered mice, and regularly review breeding so that excess animals are not produced. In some cases, wild-type animals that are not required for efficacy studies (which often only use genetically altered animals) can be used for other purposes e.g. tolerability studies.

Use of cell culture systems will reduce the number of experiments that need to use live animals. All compounds will be triaged through in vitro assays so only that only those with the most favourable profiles are progressed to in vivo disease models. We will conduct pilot experiments where necessary to ensure that our experimental systems are optimised before conducting large studies. Colleagues with expertise in drug metabolism work closely with us, and we share tissues with them 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.

Wherever, possible the late onset disease model will be used but in some cases it is necessary to assess the effectiveness of therapeutic approaches against a more rapidly progressing model that displays clinical signs. However, previous work has allowed us to develop a breeding and maintenance strategy for this line which minimises the impact of the harmful clinical symptoms:

1) The line will be maintained on a mixed genetic background (B6CBA) to promote hybrid vigour.

2) Female mice are infertile so colonies must be maintained through the male line exclusively. Breeding males have a short fertility window (5-8 weeks of age) and will not be kept beyond this age, which is before harmful clinical symptoms impact on welfare.

3) Pups will be weaned at 4 rather than 3 weeks so that they are larger and stronger when they go through the weaning process. Where possible mutants will be housed with wild-type littermates, which has been shown to positively impact on welfare and increase lifespan by a week.

4) Mice displaying clinical symptoms will have easy access to food and water - food on the cage floor and water bottles with a long spout. Extra bedding will also be provided to absorb excess urine.

5) Animals displaying clinical symptoms will be monitored closely on a daily basis, and signs of distress

e.g. sudden loss in body weight, piloerection, hunched posture, reduced spontaneous activity trigger increased health monitoring. If any further deterioration occurs, animals will be promptly and humanely killed.

6) Mice will not be maintained to an age where they experience end-stage disease.

Surgeries will use high standards of aseptic technique as well pre- and post-operative care to minimise pain and aid recovery.

Why can’t you use animals that are less sentient?

The mouse models of disease we plan to use carry the gene mutations which cause disease in patients, therefore improving the likelihood of translation from efficacy observed in these models to the clinic. An intact mammalian brain is also important for performing meaningful electrophysiology studies and analysis of more complex behaviours. Overall, mice provide the best compromise in terms of correlating with human biology whilst exhibiting the lowest level of sentience. Studies in terminally anaesthetised mice are not appropriate because we need to monitor disease progression over the course of a number of weeks.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Regular evaluations of procedures and their associated welfare implications will be conducted throughout the project. Findings will be discussed in the context of improvements that can be made without impacting on scientific outcomes. We will also seek advice from the NACWO and NVS, as well as other scientists working in the in vivo facility.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will refer to published guidelines issued by NC3Rs as well as LASA (Laboratory Animal Science Association) and ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines, to ensure our studies are performed in the most refined way.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay informed by consulting the NC3Rs website (https://nc3rs.org.uk/), attending relevant talks and conferences e.g, the NC3Rs Pint of Science events and consulting with colleagues. 3Rs information is also distributed by the Named Information Officer and the Named Training and Competency Officer. We will liaise frequently with the Named Veterinary Surgeon (NVS) and Named Animal Care and Welfare Officer (NACWO) to get advice on how to implement any advances.

93. Provision of surgically prepared animals to other projects with the authority to use them

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
- 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

Surgery, Generic Licence, Rodent

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?

This is a Generic Licence to generate standard surgical models for work carried out under current Project Licenses by commercial clients and 'in-house' research clients.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The primary benefit of the programme is that a service is provided to the scientific community where there may not be the expertise to conduct surgical modifications or where capacity to produce the required groups sizes for their work is limited. A secondary benefit is that the procedures are performed to a consistently high standard due to the routine nature of the service and the established expertise of the surgical team. To minimize the risks of animal welfare being compromised, the surgical procedures are conducted by trained and competent surgery technicians in a purpose-designed suite of rooms.

Surgery protocols are based on best practice and experience and are tailored for each type of surgery, particularly with respect to anaesthesia, analgesia, supportive therapy and postoperative care. The activities are overseen by both a Named Animal Care and Welfare Officer and the Named Veterinary Surgeon, who are available to offer advice and expertise on animal health and welfare.

What outputs do you think you will see at the end of this project?

The provision of this service will provide support for scientists that have PPL authority for the use of surgically prepared animal models in their research and development process for novel human and veterinary medications and the development of novel therapeutic procedures.

Who or what will benefit from these outputs, and how?

The general public will benefit from new drug discovery processes and safety testing as a result of the surgically prepared animals supplied to client research groups under the provision of this Project Licence.

How will you look to maximise the outputs of this work?

Surgical procedures are conducted by trained and competent surgery technicians in a purpose- designed suite of rooms. Surgery protocols are based on best-practice and experience and are tailored for each type of surgery, particularly with respect to anaesthesia, analgesia, supportive therapy and postoperative care. The activities are overseen by both a Named Animal Care and Welfare Officer and the Named Veterinary Surgeon, who are available to offer advice and expertise on animal health and welfare.

In addition, we communicate on a regular basis with other industry surgical teams to ensure dissemination of knowledge and experience.

Feedback is obtained from all clients and as a result, surgical models are under continuous refinement.

Species and numbers of animals expected to be used

- Mice: 4000
- Rats: 7500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Choice of species, strain, sex and life-stage are customer driven and dependent on the research demands of those that will use the animals, with advice given by experienced surgical technicians and Named Veterinary Surgeon where appropriate.

Typically, what will be done to an animal used in your project?

Subcutaneous injections for pre-emptive and postoperative analgesia.

Induction and maintenance of inhalational general anaesthesia and surgical procedures (e.g. soft tissue procedures, vascular or non-vascular catheterisations and cardiovascular telemetry surgery).

Based on past experience, over the period of 5 years we would anticipate using approximately 4000 mice and 7500 rats.

Examples of the types of surgery that we intend to perform include

1. Routine soft tissue surgeries such as castration, ovariectomy, vasectomy and splenectomy, including the preparation of sterile male mice for mating, to obtain pseudo-pregnant females to be used for embryo transfer.

2. Catheterisation models for dosing and sampling during PK/PD studies.

3. Telemetry models for the remote monitoring of biological signals such as heart rate, blood pressure and ECG.

4. Bile duct catheterisations for metabolic studies.

What are the expected impacts and/or adverse effects for the animals during your project?

Pain may be experienced in the immediate postoperative period but this will be controlled by the use of analgesic regimes as proposed by the Named Veterinary Surgeon.

If an animal experiences unexpected adverse effects or pain that cannot be controlled it will be humanely killed using an approved method.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

100% of mice and rats are expected to experience no more than moderate severity levels.

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?

Animals are currently essential for research into human and animal diseases, to learn about biological processes in animals and humans, to learn about the cause of diseases, to develop new treatments and vaccines and evaluate their effects and to develop methods that can prevent disease both in animals and humans.

Rats and mice are the most commonly used animals in biomedical research because they are readily available, easy to handle and very similar to humans physiologically and genetically. In addition, most of the mice and rats used in medical trials are inbred so they are almost identical genetically, helping to make the results of medical trials more uniform. Rodents also reproduce quickly and have a short lifespan, so several generations of animals can be observed over a relatively short period of time.

By using surgically prepared models, researchers can easily perform techniques such as dosing and sampling with minimal amounts of distress caused to the animal and allowing multiple time points in the same animal, reducing usage. In addition, surgical alterations can mimic human disease states and allow monitoring of biological signals within the home environment.

Bile duct cannulation studies are usually carried out in the rat to help determine the pharmacokinetic profile (absorption, distribution, metabolism and excretion) of new agrochemicals and pharmaceuticals, which is a regulatory requirement.

Which non-animal alternatives did you consider for use in this project?

Non-animal alternatives are assessed as part of the client PPL.

Before commencing any new package of work we meet with the client to discuss alternative models, species or strains and the justification for animal use is assessed further with regard to scientific background and study requirements. Any new requests for animal models are then further evaluated by our Animal Welfare and Ethical Review Body.

As an example, the use of sterile hybrid mice as an alternative model to vasectomisation requires maintenance of 2 colonies, C57BL/6J and a wild mouse sub-species, Mus musculus musculus. The latter are not easily available and are challenging to breed and therefore production of sterile male mice may not be commercially viable.

Why were they not suitable?

Behavioural models and full system responses cannot be replicated using in vitro or in silico methods.

Clients are questioned prior to commencing any new package of work to ensure that viable alternatives to the use of animals are not available, and responses are discussed by our Animal Welfare and Ethical Review Body prior to any preparation of animals.

Where animals are used in research projects, they are used as part of a range of scientific techniques that may include human trials, computer modelling, cell culture and statistical techniques, and we ensure that animals are only used for those parts where no alternative to their use can deliver the answer.

For example, the development of novel treatments for cancer (e.g. soft tissue surgery), assessment of complex whole body responses to new chemical entities (e.g. telemetry models) and pharmacokinetic/pharmacodynamic modelling (e.g. vascular and non-vascular catheterisations).

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 is based on animal usage figures for the past 5 years and current known future requirements.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The number of animals required is dictated by the client, although discussions with end-users and the relevant Animal Welfare Ethical Review Body (AWERB), along with

continuous monitoring of outcomes ensures we prepare minimal numbers of animals to support 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 using surgical best practice and regularly reviewing our procedures and the competence of our staff we ensure that only the smallest number of animals are prepared to meet the order quantities, based on experience and model specifications.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Standard, well defined rodent surgical models will be prepared under this PPL, the choice of species being determined by the client.

We ensure best practice under Veterinary guidance regarding the use of anaesthetic and analgesic regimes.

Examples of how these surgical models and methods cause the least pain, suffering, distress, or lasting harm include:

Vascular catheterisation models in combination with percutaneous skin buttons, which allow sampling and dosing in experimental animals with minimal amounts of stress and allow them to be housed in compatible group sizes.

Telemetry models, which allow the evaluation of animals within their home cage and paired with companions.

Use of buried suture patterns that allow group housing to reduce stress and reduce the number of postoperative interventions.

Why can’t you use animals that are less sentient?

The choice of species and life stage is determined by the client and justified under their PPL.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Refinements include acclimation of animals within the facility prior to any procedure including regular handling, extensive postoperative monitoring by trained personnel, including out-of-hours cover, and the use of recommended analgesic regimes for both pre-emptive and postoperative pain relief.

Other measures that we undertake to minimize welfare issues include housing in socially compatible group sizes wherever possible, and by the provision of enrichment and supplements during the recovery process.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

As well as published research papers, best practice guidance may be assessed using publications such as the ‘Guide for the Care and Use of Laboratory Animals’, ‘Laboratory Animal Anaesthesia’ by

P.A. Flecknell and the LASA 2010 ‘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 collaborate closely with global colleagues and clients to share knowledge and experience, and ensure that the highest standards are used at all times.

Regular review of information on websites such as NC3R’s, LASA, Procedures with Care. Attendance on company and external webinars and seminars.

94. Immune response in health and disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Inflammation, immune-mediated diseases, cancer, tissue damage, ageing

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of our research is to discover how the immune system recognises and responds to diseased or abnormal body tissue as occurs in inflammation, cancer, autoimmunity, ageing, and how this response relates to normal healthy tissue. We ultimately aim to identify ways that inflammatory diseases can be treated.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 our understanding of the underlying ways that the human immune response leads to tissue damage is limited, treatment is often delayed, may not be the most suitable, and only started when symptoms are already present. The development of ways of treating immune conditions in their early stages is a key future challenge and would improve patients' lives considerably. A deeper understanding of how the inflammatory/immune response of the body develops into chronic (long term) inflammation, autoimmunity and

cancer, when it is not properly controlled, will allow us to identify new treatments to prevent and reverse tissue injury.

What outputs do you think you will see at the end of this project?

The work we propose to carry out will contribute to our understanding of the extent to which the immune system is involved in tissue injury as well as how the immune response may change in older people. We hope to gain new knowledge on the mechanisms controlling the state of normal body tissue and how these mechanisms are changed in chronic inflammatory conditions affecting different organs, specifically skin and kidney, and during ageing.

The second output is the publication of our work in scientific peer-reviewed open-access journals and presentations of the findings at national and international conferences to share the newly acquired knowledge.

The findings from our work will also provide valuable information which can be used in the development of new clinical trials based on the potential treatment options we hope to identify.

Who or what will benefit from these outputs, and how?

The scientific community will be the primary beneficiaries of this project as our results will advance the current knowledge in the immunology/inflammation field as the data will be made freely available. In addition, scientists around the world will also benefit by having access to special types of mice that we will make which can be used for this type of research.

In the mid-term this research may be of interest to drug companies that wish to develop compounds capable of reducing inflammation and of preventing tissue damage. Such treatments could be used for a wide range of inflammatory and autoimmune conditions.

In the long term, discoveries we hope to make could result in economic benefits because of reduced healthcare support required as treatments will be more effective. Better treatments would significantly enhance the quality of life for patients affected by these conditions. This may happen sooner than anticipated as demonstrated by our research discoveries that have changed the lives of patients with specific kidney diseases who as a result no longer need dialysis or kidney transplant.

Additionally, medical charities (e.g. Versus Arthritis, Cancer Research UK, Wellcome Trust) may be influenced by our studies as this programme may inform funding priorities.

How will you look to maximise the outputs of this work?

We will work with other groups, seeking advice and expertise where necessary. We will also join forces with pharmaceutical companies that have new drugs to test.

Our knowledge will routinely be distributed to our colleagues, including clinical personnel, via monthly research meetings, as well as to other members of our faculty interested in immunology/inflammation through inter-department talks. We will present our findings at national and international conferences to further share our knowledge across the world. We will publish our work to share our findings with a broader range of interested scientists

and clinicians. We also work very closely with the charities who fund our research and regularly present our progress to them, which we will continue to do with this project.

Finally, any research tools developed in this programme, any post-mortem tissue samples, or data we gather, will be made available to other scientists working in the same field or in drug development.

Species and numbers of animals expected to be used

- Mice: 40000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 our studies for the following reasons:

Most of the immune genes in humans have an equivalent in mice and genetically modified mice are available which closely mimic human disease to enable us to achieve the aims of our work and to apply our findings to human disorders.

The broad availability of experimental reagents enables us to investigate more deeply the systems on which our work focusses; this in turn allows us to reduce the number of animals needed.

Mice are widely used in research and there is already a large amount of information available to us which we can refer to.

We will mainly use adult mice because our research focuses on conditions generally present in adults. In adult mice the immune system is fully developed and experimental results tend to be more reliable. In rare situations, we may need to use juvenile (very young) mice to make some changes to their genes before the immune system is fully developed.

Typically, what will be done to an animal used in your project?

We will try to replicate (in mice) some of the inflammatory conditions seen in patients, using well established experimental designs. Mice will be subjected only to one of the protocols (experiments) outlined below.

Protocol 6 - Animals may need to have blood or bone marrow or urine samples taken and be injected with a substance which will bring about an immune/inflammatory response. Injected substances will be at the lowest dose possible to bring about this response and ensure minimal suffering. Animals will be fully recovered between injections which may cause them to experience mild, transient pain but no lasting harm from this. There will be no cumulative effect from repeated injections. Some animals may have devices implanted under surgery which may cause some discomfort afterwards with mild to moderate pain which will be treated with painkillers. Some animals may have substances applied to their skin, others may be given substances in food or drinking water. In every experiment the

less harmful route of administration will be used. On rare occasions (<5%) animals will have a transplant (e.g. bone marrow) before the experiment starts. In less than 5% of the experiments animals will be fed a special diet which should not cause distress but may sometimes result in obesity or itchy skin, or weight loss. Animals will be placed onto normal diet should they lose 15% of their body weight.
Occasionally (<10%) we will also use older (>80 weeks of age) mice and compare their immune response to young mice. The duration of the experiment varies according to the substance injected – most experiments last 3-4 weeks, but some may continue for several months.

Protocol 7 - Some mice (<10%) will be subjected to procedures that target the kidney as kidney damage is one of the main causes of death in patients with immune-mediated conditions. In most cases the mice are injected with a kidney-damaging substance. On rare occasions (<5%), animals will have a transplant (e.g. bone marrow) before the experiment starts, or be put on a special diet. The animals will be humanely killed at the onset of signs of kidney damage. In some circumstances we may have to collect urine by placing single animals in cages covered with special non-absorbent sand or in specifically designed cages for up to 18 hours (typically overnight) with free access to food and water. Mice will be singly placed in cages no more than twice a week. Blood samples may be taken every 2-3 weeks. The mice will be humanely killed as soon as we see signs of kidney disease from changes in blood and urine. The duration of the experiment is usually 1-2 weeks, but some experiments may continue for several weeks.

Protocol 8 - The skin is continuously exposed to a wide range of potentially harmful substances present in the environment, including chemicals and ultraviolet (UV) irradiation (artificial sunlight). Skin damage is one of the most common clinical manifestations seen in long-term conditions. In this protocol, each of our experiments mimics a specific human condition and will damage a limited area of the skin. In most cases this will be done to an animal only once and, if necessary, under anaesthesia.

Blood samples from the animals will be regularly checked afterwards for signs of disease. On rare occasions (<5%), animals will have a transplant (e.g. bone marrow) before the experiment starts. In less than 5% of the experiments, the animals will be fed with a special diet to assess the effects on the skin or we will use older animals (>80 weeks of age). The duration of the experiment is usually 1-2 weeks, but some experiments may continue for several weeks.

Protocol 9 - It is known that inflammation triggered by exposure to environmental agents such as UV rays in sunlight plays a key role in the development of skin cancer. We plan to bring about the growth of skin tumours by applying substances present in the environment to the skin of mice. On rare occasions (<5%) mice will be injected locally with tumour-forming cells, but tumours will not be allowed to grow to the point of where they can cause suffering to the animals. The animals will be regularly checked and assessed for overall health in several ways previously agreed with the vets and Named Animal Care Welfare Officers. Great care will be taken to ensure we detect tumours as they develop. In less than 5% of the experiments we will feed the animals with a special diet to assess the effects on skin cancer development and may expose mice to other substances so that we can look at the effects. Wherever possible the substance will be administered in the diet or drinking water or injected. Blood and/or urine samples may be taken. The presence of tumours will be limited to the minimum required for useful results. In all cases, the general health and condition of the animal will remain the overriding concern. The duration of the

protocol varies according to the tumour-forming substance applied but, in most cases, it lasts approximately 20 weeks.

What are the expected impacts and/or adverse effects for the animals during your project?

The experiments planned under this licence involve procedures which are not expected to cause the mice severe distress or discomfort. Mice may have injections which are expected to cause mild and short-lived discomfort, although some mice may develop reduced appetite/eating less or ruffled fur. All the mice will recover from these symptoms within 24hrs. Some injections may lead to inflammation at the injection site and injections into the belly area may lead to mild tenderness and swelling for a few days with no signs of distress. Mice that have injections into muscles may limp after for a few hours and may experience some pain.

A small proportion of animals (approximately 10%) will undergo moderate surgical procedure which will be performed under general anaesthesia with pain-relief during and afterwards if necessary. Some animals (approximately 30%) in tumour experiments will have slow-growing tumours appearing after about 10 weeks. These animals will be checked daily for signs of harm and humanely killed before they experience severe distress.

Approximately 15% of animals may show kidney damage, but we can easily identify this from urine samples, and mice will be humanely killed as soon as signs of disease are seen and before they develop signs of ill health.

Less than10% of animals may have some physical abnormalities because of their genes being altered. These animals will be closely checked for these from an early age and humanely killed before they develop signs of ill health.

Animals more than 18 months old (< 5%) may develop tumours or seizures due to old age. Old mice will have enhanced welfare checks at least weekly; mice will be humanely killed before they develop signs of ill health or distress. All procedures have been designed to be stopped as soon as the animals appear to be suffering.

In all experiments we will monitor the condition of the animals regularly according to the protocol used. If unexpected distress occurs, 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)?

Overall, for this project, it is estimated that 30% of the mice will experience subthreshold severity, 40% mild severity and 30% 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 immune response is a complicated process that involves a network of movement of very small substances in the body which cannot be studied outside of the living organism or by using computer simulations, so experiments using intact animals are essential to study the immune response. The protocols described in this programme of work aim to mimic what happens in human conditions in which different responses inside the body play a key role.

Although we can test some aspects of the immune response in the laboratory using different techniques, we are not yet able to fully reproduce the complexity of the human immune system or the response of the different organs in their entirety. Experiments with mice are the only way of testing ideas from our laboratory work and of studying how effective potential new treatments might be. Our experiments would not be possible with fish or insects as their immune responses do not act in the same way as those seen in human disease.

Which non-animal alternatives did you consider for use in this project?

We have very carefully considered if we can achieve the same aims by using non-animal or computer- based methods All the planned animal work will only be carried out after intensive non-animal laboratory experiments which help us find out whether animal experiments are necessary and likely to work. Where possible we will use samples from patients and heathy volunteers rather than use animals. Furthermore, over the duration of the licence we will continually look for ways to replace

animal with non-animal experiments, particularly once we know how the genes of interest work in the animal. Finally at the end of each procedure, we will use blood and tissue samples taken from the animals to gain as much information as we can.

Why were they not suitable?

Although non-animal techniques can provide important information, they cannot fully reproduce the complex processes inside a living animal; inflammation and/or tissue damage are processes involving the whole organism which cannot be fully reproduced. We require the complexity of an animal to fully reproduce these conditions, so that we can properly understand how the immune system responds to substances, environmental factors and ageing. Computers are not yet smart enough to be able to simulate every possible reaction that a cell or animal could have. Therefore, animal models remain the only way of testing theories based on non-animal studies and ultimately studying the effectiveness of potential new treatments.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers estimated here come from our long-standing experience in the different protocols. Our programme of work has several aims which we plan to achieve over a 5 year period, and the proposed number of animals is our best estimate of the number necessary to achieve these aims. Sample sizes for our experiments are estimated from past experiments. Calculations typically show that we need group sizes of 5-8 to achieve the quality of results we need. We have also the numbers of animals used in previous licences to estimate the number of animals that we will need to use for breeding.

We estimate that a maximum of 8000 mice per year might be used. However, this is an upper estimate and the actual number used is likely to be very much less.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will continue to design and perform experiments following general principles of good experimental design and laboratory practice following the ARRIVE guidelines. Where possible, we will explore multiple aspects simultaneously, so that control groups (animals not undergoing experiments, used to compare with those undergoing experiments) can be shared between experiments/research groups, reducing the total number of mice required. Where we can, we will use experiments which give more reliable results with fewer differences between female and male mice which means we will maximise the use of existing animals. We will maximise the amount of data we can acquire from individual mice by monitoring the disease using blood or urine samples or non-harmful imaging (special photography) methods. In most of our experiments, we will take samples from different organs after the animals have been humanely killed for further testing. This means we don’t need any more experiments just to obtain such samples, reducing the total 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?

The following measures will be taken to optimise the number of animals in our project:

We routinely use a computer-based system (GraphPad Prism) to estimate numbers of animals and will consult in-house Statistical Advisory Service, if necessary, for statistical analysis of animal numbers needed for our studies.

Experiments will be conducted using well-matched groups. To avoid bias and variability, we will allocate the mice to each experimental group in a random and blind manner. If possible, we will generate initial data using very small experimental groups (2-3 mice/group)

Mouse colonies will be closely monitored to avoid excessive breeding. We will freeze sperm/embryos to reduce the need for maintenance of genetically modified mice while preserving them for future use.

Before we generate new genetically modified mice, we will ensure that this type of mouse does not already exist by checking mouse locator services and by searching mouse

databases and publications, e.g.the NC3R's mouse database, Jackson Laboratory database or Cre transgenic database.

In most experiments, organs will be taken from dead animals at the end of experiments for further tests, reducing the total number of animals required. The samples will be shared with other groups and may be used for further experiments in the laboratory.

Whenever possible, in accordance with 3Rs guidelines, we will use alternatives to genetically modified animals such as administration of vectors like adeno-associated viruses expressing proteins. The use of AAV mediated transgenesis will avoid the generation of genetically modified animals by conventional transgenesis, thereby significantly reducing the overall number of animals.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We only intend to experiment with mice.

We will use both wild type ('normal') and genetically modified mice. We will study mice with genes which have been altered in such a way the animal well-being is not affected. Most of the genetic changes that we make in mice cause no pain, suffering, distress, or lasting harm. We may give or apply substances (e.g tamoxifen) to cause the genetic modification, using established treatments that do not themselves cause pain, suffering, distress, or lasting harm. For example, local AWERB guidelines for administration of tamoxifen will be followed.

To bring about immune/inflammatory responses we will give or apply substances in accordance with good practice guidelines and will follow our previous experience using similar types of mice and/or available literature. Doses of substances and duration of dosing will not exceed those reported to have the desired scientific effects in the literature. LASA guidelines will be adhered to, and the most refined method will be used. To reduce risk of accidental infection, sterile techniques will be used.

The proposed experiments will be carried out in such a way that the animals endure inflammatory effects for the minimal time required for our studies.

The tumour models we will use don't spread throughout the animal.

Whenever possible, we will use experiments which produce results over shorter periods of time.

Why can't you use animals that are less sentient?

Mice remain the most valuable and appropriate species for our studies and the reasons are the following:

- We cannot use species that are less sentient, such as insects or fish, because these animals do not allow us to model the immune/inflammatory response seen in humans.

- Mice are the lowest vertebrates where genes can be readily altered and thus represent an ideal model to study the processes we are researching.

- The mouse immune system shares similarities with the human immune system and therefore allows for comparison to human disorders.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Different measures will be applied to minimise the harms for the animals. These are listed below:

In all procedures, we will monitor the condition of the animals regularly. If unexpected distress occurs, mice will be humanely killed using a method authorised by this licence.

Where there is a risk of whole body illness (i.e. not isolated to a single organ), the frequency of monitoring will be increased.

The animals will be group housed and environmental enrichment will be provided to improve animal welfare.

Where there is a risk of pain, analgesia will be provided in advance, as advised by our Named Veterinary Surgeon (NVS).

When the repetitive use of a substance is required, wherever possible, the administration will be carried in water or food, or by applying to the skin, or via a pump or a slow-release pellet to avoid use of repeated injections

Whenever possible, we will use experiments which produce results over shorter periods of time.

Where recovery from surgery is required, the animals will be placed in a warmed cabinet and pain relief given following advice of the NVS. All animals will be closely monitored after surgery and any animal showing signs of poor recovery that fails to respond to prescribed treatment or whose condition deteriorates will be humanely killed

Anaesthesia, where used, will be of depth sufficient to prevent the animal being aware of pain from the procedure.

Experimental methods, including dosing and sampling volumes and frequencies, will be in accordance with current best practice (using sources such as LASA).

Ageing animals will be carefully monitored by staff trained to work with them. Group sizes in ageing experiments will be increased to accommodate for loss of animals and to avoid housing animals alone. Animals will be monitored for adverse effects such as changes in weight, dermatitis, piloerection (raised fur), paleness, changes in mobility, lumps, eye

defects, abnormal breathing, or stools. If these are observed animals will be treated accordingly, and animals will be humanely killed before they develop any signs of distress.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will adhere to guidelines issued by LASA and NC3Rs and will endeavour to report our findings accurately using ARRIVE and PREPARE (Smith et al Laboratory Animals 2018, 52, 135–141) guidelines. In addition, we will use the guidelines set by our institution to ensure the experiments are designed and carried out in the most refined way possible.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Institutional seminars and workshops will provide continuing professional development in the 3Rs, while the NC3Rs website always provides a readily available resource.

To effectively implement the 3Rs in our work, we will discuss these issues regularly at our group meetings so all researchers who carry out animal work are kept informed on how to maintain best practice.

95. Generating and Maintaining Zebrafish Targeted Mutants and Crispants

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

Genome editing, CRISPR technology, Zebrafish, Development, Disease

Animal types	Life stages
Zebra fish (Danio rerio)	embryo, neonate, juvenile, adult, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to create a resource/technology for zebrafish, that can be used to modify a protein quickly and precisely in the genome to switch it off or on, or in order to create zebrafish "carbon copies" of human diseases, this can be done using the so-called CRISPR-technologies. In addition, we want to develop this technology, to label proteins precisely with tags in the genome such that their role can be studied in high detail.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Advanced biological research often leads to lists of candidate-genes that may be involved in a particular biological process. For instance, certain genes may cause, worsen or improve a disease, or they might participate in a particular developmental process. An example might be the formation of an eye; a study might identify a list of genes that are specifically active while the eye is forming. However, proving that such a candidate-gene is really essential, requires extensive genetic procedures and a lot of animals, in order to block the function of that gene and the protein that is produced by it. A genetic technology named CRISPR, can now be used to make such follow-on tests much more efficient and precise, thus our work will benefit many scientists working in a variety of fields.

What outputs do you think you will see at the end of this project?

We will have created efficient protocols to apply CRISPR technology in zebrafish (CRISPR is a technology that can be considered as highly precise molecular scissors that can be used to cut the genome of an animal at a very particular position). In particular, we will develop protocols to manipulate bases more precisely. Rather than creating a random change in the DNA, we hope to develop and improve methods to create precise changes. For instance, if in humans one particular change in a protein causes a disease we will be able to recreate exactly the same change in zebrafish thereby creating the "best possible" disease model. We will publish our results in the scientific literature.

Who or what will benefit from these outputs, and how?

In the short term we will test and develop new and better ways to apply CRISPR to the zebrafish, to modify their genome in a directed fashion, this is currently not easily possible. Having this technology, will allow us to create better models of human disease that more precisely mimic the genomic changes that happen in patients with a genetic disease, an thus could allow us to study precisely why particular genetic mutations cause disease whereas others don't. In the long term it could help to discover drugs that are precisely tailored to patients. Once worked out, other scientists may also benefit from adopting our technology.

How will you look to maximise the outputs of this work?

We will collaborate with relevant scientists in the department and beyond. We have already given a workshop and a webinar on the technology, we will continue to do this.

We will publish our results in peer reviewed scientific journals as we have previously done. We are currently preparing a manuscript on precise genome editing.

We will use genes and mutations that are relevant to our current scientific interest, which are currently DNA repair and cell signaling. Therefore, if our experiments are successful we will produce alleles that are directly relevant to our research rather than "poster cases". Where relevant, we may collaborate with other scientists to create GA fish in further relevant genes.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 14500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 most cases we will work on embryos, therefore adult fish are only required in natural matings to generate these embryos. Occasionally, to identify the correct genetic type of animal, we will take a small part of the fin or do a skin swab, this does not significantly impair fish wellbeing. In some cases, fish may be anaesthetised if they are required for e.g. genetic tests, observation, or recovery of unfertilised eggs from females. Anaesthesia may cause light transient bleeding from the gills. Rarely, fish may have difficulty waking up or very rarely they may fail to recover at all. In a few cases we may create an older animal that have an altered gene of interest to see what the effect of this is on the adult. Such animals will be monitored carefully, and they will be euthanised if their well-being is significantly affected, for instance if they have difficulty maintaining posture, difficulty feeding or an open wound.

Typically, what will be done to an animal used in your project?

In most cases eggs will be injected with one or more CRISPRs targeting a gene. If they are very effective, they may block the function of that gene completely, and the resulting larvae are called CRISPants. The effect of this block on CRISPant development will be studied, in most instances the CRISPants will be analysed at larval stages, before they become protected by law, and only if these larvae look healthy we may raise a small fraction to older stages. Health status will be determined visually the shape and behaviour of the larvae should be normal. An important "reporter" of the health status of a larva is the swim bladder which should have air in it 5 days after fertilisation, when larvae become protected by law. Only larvae with air-filled swim bladders may be raised.

Eggs receive a complete copy of the genome from both parents. In other experiments we will use a lower dose of CRISPRs, the resulting fish have only one copy of the gene altered and this means they are healthy and develop normally, as they have a normal copy left. Such fish can be raised and bred to create stocks of animals that are have a genomic alteration of choice for further breeding and creation of larvae that have both copies of the gene altered, such larvae are called mutants. We will only raise fish that do not have significant health issues in such cases. Health status will be determined at the age where the larvae become protected by law, and we will use the criteria as mentioned above; apparent normal shape and behaviour and the presence of an air-filled swim bladder.

In some cases it will be essential to raise animals with an induced mutation to adulthood, or in some cases to age them to some degree. This is to do an initial verification to determine if the effects of a disease causing mutation in zebrafish correspond with effects in patients. For instance, if a particular mutation causes cerebellar degeneration in human patients it will be essential to verify in the same occurs in a equivalent mutation in the zebrafish. This for instance involve some testing of behaviour, or morphology of such animals.

What are the expected impacts and/or adverse effects for the animals during your project?

We are not intending to grow animals with significant health issues, but unexpected effects can always occur, because we make new genomic alterations in genes that may have novel functions. If such significant effects on the health of the fish are observed will euthanise the animal. This will be for instance, if a fish has difficulty maintaining posture or has difficulty feeding.

Fin clips or skin swabs may be taken but these will at most result in short (<1day) mild discomfort. In some cases, fish may be anaesthetised if they are required for e.g. fin clips, swabs, observation, or recovery of unfertilised eggs from females. Anaesthesia may cause light transient bleeding from the gills. Rarely, fish may have difficulty waking up or very rarely they may fail to recover at all.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Most animals will not have significant effect on their health. In about half of the animals we will use genotyping methods to identify fish of the desired genotype, this involves swabbing or taking a small amount of fin material for analysis, and this is classified as a mild procedure, We may get some fish that due to the mutation of interest may display a mild phenotype but this will be in less than 25% of the animals.

We expect the other fish to be "sub-threshold" as they are only used for breeding to produce embryos of interest.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Our long-term aim is to create CRISPR resources and technologies which can be used to precisely modulate every gene in the zebrafish. The resulting mutants will be used to create animal models of human diseases or to understand the role these genes play animal development. This can only be done properly in the context of an animal embryo.

Which non-animal alternatives did you consider for use in this project?

We have considered in vitro methods but the nature of our work makes this difficult/impossible. We have looked in PubMed ("vertebrate development" "in vitro" "embryoid") in order to identify in vitro methods. Importantly, the choice of our gene of interest will always be based on in vitro, or patient work, that has identified that gene to be a candidate for a disease or a particular developmental process. We also considered lower animals, like fruit flies.

Why were they not suitable?

In vitro, methods have their limitations. Defects in gene function often have consequences that are not direct but are a result of interplay between different tissues, for instance nerve and muscle. Similarly, diseases are often the end result of multiple interacting processes, rather than based on a single defect in a cell. For instance, a tumour is the result of interplay between cancer cells blood vessels and the immune system. Such conditions are difficult to recreate using cells grown in the laboratory, and the in vivo context is still required. There is some progress in creating "embryoids" from stem cells in mice, but these do not develop very far at the moment. Therfore, it is currently impossible to study complex interactions that are required to build an embryo or larvae in vitro. For lower animals, the anatomical differences, like absence of blood, blood vessels and a vertebrate immune system, makes these less relevant.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 numbers are largely determined by stock-keeping requirements which dictate that a minimal number of adults are required per line to ensure availability, and capacity. Where live stocks of mutant lines are not continuously required, they will be stored as a frozen as

sperm sample. We have estimated from our previous work in CRISPRi that we would maintain 20 lines over 5 year and the generation of 10 new lines per year, this will require 12000 fish over 5 years. In addition to maintain lines or freeze lines we estimated we might need to generate oocyte or sperm from 500 animals

In a few cases we may need to use adults/older larvae directly in experiments, here we will calculate to minimal number required to obtain answers to the scientific question posed. We expect to be raising about 10 goups of animals to test adult phenotypes per year; this is estimated to involve 2000 animals over 5 years.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

As stated above, in most cases fish are used to generate the eggs that we will work on. We will minimise the number of these fish in our stocks by doing genotypic or other selection experiments as early as possible. For instance, do DNA or other tests to identify the correct fish before the age of legal protection and only raise the correct animals.

Where older animas are required, we will design a plan, and use experimental design software, like NC3R's Experimental Design Assistant, to minimise the number of experimental animals.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Animal stocks that are not required will be frozen as sperm samples.

Our aquarium facility has optimised care of our fish and fish can be kept fertile and healthy for 30 months, this will keep the number of stocking fish required to maintain a line low as less generations are required.

In addition, by development of efficient CRISPR technology, it will be possible to create fish that have a defective gene using egg injection rather than maintaining stocks carrying a defect in a gene of interest. Thus it could lead to less stocks being kept. It also could avoid shipment of stocks to other labs. Instead, information on "what CRISPRs to use and how" can be shared electronically.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 choice of animal also reflects refinement, since fish are the simplest model vertebrate in which these studies can be performed, and our experiments are on embryos or larvae which have a lower level of awareness relative to adults. Most of our experiments will be done with embryos less than 5 days old, and thus will fall outside the Animals (Scientific Procedures) Act. Our CRISPR resources will increase the appeal of zebrafish relative to mammals.

Why can’t you use animals that are less sentient?

For the great majority we are using immature life stages, we will only use older animas when our experimental aims cannot be achieved otherwise. When we use these animals we are taking advantage of their less complex neurophysiology compared with higher vertebrates (e.g. mammals).

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Most of our animals will not undergo significant procedures, occasionally fish may have to be anaesthetised to do do more detailed observations. These fish will be closely observed to ensure they have recovered well.

During finclipping we will only remove the very tip of the tail, and analgesia will be provided.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will work according to the suggestions provided by Aleström et al. 2019 with respect to fish care.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We are taking regular courses offered by NC3Rs. We have regular meetings with the Aquarium team within the our establishment where new developments are communicated. Any experiments using protected animals will involve writing a plan which will be discussed with the Aquarium team to minimise harm and to provide further input on the best possible approaches. I am actively involved in our Ethical Review Committee and get additional information via this channel.

96. Dietary fibre and manipulation of the gut microbiota to improve outcomes in pelvic 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

Radiotherapy, Radiosensitisation, Normal tissue effects, Pelvic cancer, Dietary fibre

Animal types	Life stages
Mice	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to identify dietary fibres, bacterial combinations and metabolites which can be used either alone to shrink tumours or in combination with radiotherapy, to make the radiotherapy more effective at controlling tumours while minimising normal tissue effects. This will improve patient survival and quality of life, and in some circumstances delay the need for definitive treatment (e.g. men with prostate cancer on active surveillance).

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 approx 375,000 new cases of cancer diagnosed in the UK per year and prostate, bladder, endometrial and colorectal cancer make up 30% of cases, with an combined overall mortality rate of 32%. Treatment options include surgical removal of the tumour and radiotherapy-based treatments, each with different side effect profiles. There is therefore scope to improve outcomes in pelvic cancer patients, both in terms of tumour cure and minimising side effects of treatment. Conventional chemoradiation treatment causes significant side effects, and is not usually tolerated by elderly cancer patients. With an ageing population, there is an urgent need to find alternative approaches. Targeting the gut microbiota through dietary fibre manipulation is one such approach. The use of a dietary fibre supplement would be significantly cheaper than chemotherapy and would reduce overall treatment costs if it reduced morbidity. Furthermore, men on active surveillance for their prostate cancer may benefit from a delay (which could even be life-long) to receiving definitive treatment with surgery or radiotherapy.

What outputs do you think you will see at the end of this project?

We expect to generate new information on the benefit of specific dietary fibre supplements and bacteria/groups of bacteria which delay tumour growth and are radiosensitising to tumours while sparing normal tissues. This information should allow us to start clinical trials in cancer patients, with the aim of achieving patient benefit.

We shall publish our findings in peer reviewed journals and present our findings at national and international conferences. Our microbiota and metabolomic data will be deposited in open access repositories.

Who or what will benefit from these outputs, and how?

There will be short-term benefits to scientists investigating the function of the microbiome and researchers investigating the control of tumour growth, expansion and development.

In the medium term, patients will benefit from entry into clinical trials (which is known to improve outcomes, even in placebo arms, due to the increased levels of care/attention received).

In the long term, the development of new nutritional approaches will augment the therapeutic efficacy of radiotherapy and/or chemoradiotherapy, with reduced side effects, and result in potential treatment of cancers in patients that are currently unable to tolerate 'standard of care' treatment. Improvements in tumour and symptom control are likely to lead to improved patient health and a reduced cost and burden on the NHS.

How will you look to maximise the outputs of this work?

Our work involves the study of small tumours under the skin, and we will be determining which fibres and bacteria/bacterial combinations are worth pursuing further in more complex models elsewhere. For example, we are already collaborating with another centre which has developed an orthotopic colorectal model.

We will work with local gut microbiota experts to develop our own mini-microbiota model *in vitro*, which could be used by other groups *in vivo*.

We are actively engaging with other cancer centres to build up collaborations, and may be able to expand our work to other non-pelvic tumour sites, e.g. breast cancer, brain tumours. We will be able to share tissues from our mice with others.

We will present our work at national and international conferences, publish in open-access journals and hold PPIe events to disseminate our new knowledge.

Species and numbers of animals expected to be used

- Mice: 2800

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are using mice as they are the least sentient animal that yields meaningful results on tumour response to treatments that can be used to take such treatments into clinical trials in patients.

Furthermore, there is significant overlap of their gut microbiota with humans, which allows us to determine the effects of dietary manipulation which may also be effective in human subjects.

We will use juvenile and young adult mice, as experiments take some weeks to complete and we do not want to study aged animals. For microbiota experiments we wish to use younger mice as we are more likely to successfully colonise their intestines with the bacteria.

We may use CD1 nude mice for experiments using human tumour cell lines, and germ free mice for bacterial gavage experiments.

Typically, what will be done to an animal used in your project?

Typically, a mouse will be started on a specific high or low fibre diet at the same time as, or shortly before, having tumour cells injected into the flank under the skin, and growth of the tumour is assessed by measurements two or three times per week. Once the tumour reaches approximately 100 mm^3, the tumour will be irradiated under general anaesthetic and the mouse will have its weight and the tumour size recorded several times per week and the mouse will be killed before the tumour reaches 1,000 mm^3 (usually by 800 mm^3). Blood will be taken under terminal anaesthesia and tumour and tissue harvested and frozen down for *ex vivo/in vitro* experiments.

Some mice may be gavaged with different bacteria/groups of bacteria to see the effect on tumour growth and radiosensitivity, and some may be treated with antibiotics or a metabolite associated with the gut microbiota. In some cases, a cellular marker such as dextran-FITC, BrdU or pimonidazole may be injected by an appropriate route up to 24 hr prior to killing.

Some mice, which may be tumour-bearing or not, after two weeks of diet, will have their lower abdomen irradiated with a single fraction of 10 to 15 Gy irradiation and killed 3-5 days following irradiation to assess normal tissue effects in the intestines.

Animals may stay on study for up to 6 months.

What are the expected impacts and/or adverse effects for the animals during your project?

Injection of tumours should be no more painful than a subcutaneous injection. Mice will be irradiated under general anaesthetic to minimise distress. Mice may experience transient weight loss of <15% after irradiation on some diets. Rarely tumours may ulcerate following irradiation and mice would be humanely killed if they develop a wet ulcer or worsening clinical condition with a dry ulcer.

Gavage is expected to be well tolerated if administered by an experienced operator. Antibiotics may result in metabolic changes due to alterations in the gut microbiota and may be associated with weight loss and poor oral intake.

As the adverse effects are covered by humane endpoints, the mouse would only suffer from these for up to a few days.

Anaesthesia may cause death in up to 1% of mice.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

While protocols are set at moderate severity, most mice will experience mild severity >95%; moderate
<5%.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We need to use animal models to test the efficacy of fibres, bacteria/bacterial combinations and metabolites at physiologically relevant doses as potential means of tumour suppression, and as tumour radiosensitisers which can also protect normal intestines from radiation damage. Effective approaches could then be taken into human clinical trials in patients.

Tumour cells exist within a complex, interconnected dynamic tissue microenvironment, including the host vasculature, immunological and stromal response, which affects response to therapy, and this can only be effectively recapitulated *in vivo*.

Which non-animal alternatives did you consider for use in this project?

We considered use of tissue culture, *in vitro* bacterial assays and human clinical trials and fruit flies.

We do already test bacteria and groups of bacteria in cell proliferation assays (MTT) and radiation clonogenic survival assays. We intend to carry out such experiments to select suitable candidates prior to mouse gavage during this project, to minimise the use of mice to only those candidates likely to show efficacy.

Why were they not suitable?

Tissue culture is not suitable as tumour cells exist within a complex, interconnected dynamic tissue microenvironment (see above).

in vitro models cannot be used to fully assess the normal tissue response to radiation (nor indeed lower organisms such as zebra fish or non-protected species, including fruit flies), due to the complex cellular and tissue responses involved, which again can be tested *in vivo*.

Fruit flies have a tough outer cuticle which makes irradiation impractical.

Furthermore, testing in species with a lower neurophysiological sensitivity (e.g. zebra fish) is not considered sufficient to support/warrant testing in man. Evidence of antitumour efficacy in mice or other rodents is generally considered a necessary step before proceeding into clinical evaluation of therapeutic agents.

We are already undertaking limited human studies: We are testing inulin and psyllium in 42 healthy human volunteers but can only take blood samples and cannot take post-mortem tissue (as for mice) or biopsy tissue for mechanistic studies, and volunteer studies are very costly. We are also collecting baseline faecal samples from radiotherapy patients but have not yet started undertaking intervention studies, as we need preclinical evidence to satisfy funding bodies.

To study effects of dietary fibre manipulation in radiotherapy patients requires large numbers of participants, due to the inherent variability of humans, unlike inbred mice, where much smaller group numbers are required. Again, there are limitations to mechanistic studies, due to the inability to obtain tissue routinely.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have used our past experience of tumour take rate (to allow for mice whose tumours cannot be analysed) and treatment effect sizes and variation to determine the number of mice required to achieve a large effect size (small effect sizes requiring a large number of mice are not considered clinically relevant or appropriate for further study). We enrol the help of a statistician to assist with calculations based on our previous results. Calculations for tumour experiments to date typically show that we require 8 mice for irradiation groups and 4 mice for non-irradiated controls but for some cell lines we use 10 and 5 mice respectively, to allow for failure of tumour take in some animals.

We have extensive experience of the normal tissue experiments and have found 6 mice per radiation dose (with three mice as non-IR) controls to be sufficient to demonstrate statistically significant worsening or reduction of crypt toxicity. These experiments are very time-consuming post-mortem, taking three researchers 5 hours per set of 21 mice to process the fresh tissue.

Pilot studies using smaller numbers of animals will be performed prior to decisions on definitive studies.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We calculate the minimum numbers of animals to be used while ensuring that the results are statistically significant. Where tumour take rate is 100% in a cell line, we will be able to reduce the number of mice, e.g. from 10 to 8 (see above) but may wish to study 10 mice where we are looking for responders and non-responders within the group.

We will use the NC3R's Experimental Design Assistant and guidance where appropriate and obtain advice from our statistician regarding randomisation and blinding, sample size calculations and appropriate statistical analysis methods.

Mice will be randomised to treatments/diets/irradiation using Excel rand() command, individually for females, and in cages for male mice (blocked randomisation).
The study will not be fully blinded with respect to tumour measurements *in vivo*, but *ex vivo* tissue analysis will be blinded.

Factorial designs will be used where appropriate, to maximise information from a minimum number of animals. For example, we will look at different cancer types in different sexes or strains, to determine different response rates to radiation/diet/bacterial gavage thus increasing the generalisability of our results. In the more complex experiments, e.g. where not all animals can be irradiated in the same session, block designs will be used to adjust for variability across the weeks of the experiment, and block designs will be used where both male and female mice are used.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Where practicable we will consider using inbred strains of mice to minimise inter-animal variability, and therefore experimental variability.

To control variability, mice to be used for an experiment will be ordered from the supplier in sufficient numbers at the same time. Male mice will be maintained in family groups by the supplier before dispatch to avoid fighting.

We will undertake pilot studies to determine tumour take rate and tolerability of antibiotics and metabolites to minimise wastage of animals.

We will create tissue banks of tumours, organs, bloods for use in *ex vivo/in vitro* studies, and share these tissues with others where appropriate.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use juvenile and adult mice, injecting tumours into their flank and only allowing them to grow to 12.5 GMD (geometric mean diameter =equivalent to 10x10x10 mm^3= 1000 mm^3) so the tumours are likely to be well tolerated. Mice will be irradiated under anaesthesia. Substances will be delivered by oral gavage or injection.

Some juvenile and adult mice will have their lower abdomen irradiated but will be sacrificed at 3-5 days before the onset of the ionising radiation gastrointestinal syndrome. Procedures are expected to be mild in the vast majority of mice, based on our annual returns' figures.

We will mainly use immunoproficient C57BL/6 and FVB mice to study mouse tumour allografts but where human tumour cells are studied, we will use CD1 nude mice which are immunodeficient (in individually vented cages).

Irradiation doses will be delivered in less than 10 minutes at doses which are known to be well- tolerated in this tumour models.

Why can’t you use animals that are less sentient?

Drosophila (fruit flies) have a less complex gut microbiota and their circulatory system is different to that of mammals. Furthermore, their carapace is resistant to irradiation.

We would not wish to use embryos or pre-weaned animals, as experiments would last until adulthood due to the time required.

Furthermore, non-mammalian animals are not considered sufficient to support/warrant testing in man. Evidence of antitumour efficacy in mice or other rodents is generally considered a necessary step before proceeding into clinical evaluation of therapeutic agents.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Pilot experiments will be carried out on any new cell line or metabolite/antibiotic without adequate data in the literature and for work involving the microbiota. These will be undertaken in Protocols 1 and 2 and will ensure that mice remain healthy in experiments under subsequent protocols. If we need to use cell lines or antibiotics not confirming to the licence, we will need to amend the licence appropriately. All cell lines will be checked for provenance, genetic identity and freedom from contamination with infectious agents, e.g. mycoplasma. We will liaise with our health screening providers to test cell lines for specific pathogens which might survive in the cell line. Mouse cells lines will be expanded from frozen stocks in tissue culture hoods using aseptic techniques to ensure sterility.

The mouse models that we use are standard in the field. All mice will be maintained in IVCs under barrier conditions to avoid infections.
Subcutaneous allografts/xenografts will be used. They have the advantage of not affecting the physiology of organs in the body. Tumour burden at the start of treatment will be 50-300 mm^3, generally 50-150 mm^3, and will be limited to the minimum required for a valid scientific outcome, with maximum permitted size set at 12.5 mm GMD (1000 mm3:GMD =cubed root of LxWxH, V=LxWxHx p/6). We have found in some xenograft models, e.g. RT112, that there is a reliable growth pattern from 50 mm^3, which allows mice to be killed well before 1000 mm^3 to yield meaningful results. However, this is not the case for some syngeneic models, where tumours need to be over 100 mm^3. Signs or symptoms of a large tumour burden are rarely seen when tumours are maintained below the maximal permitted size.

In our radiation experiments, mice will be irradiated under inhalational anaesthesia to minimise stress and pain. We aim to minimise the radiation dose to surrounding normal tissues which are not of experimental interest. We can use, for example, collimation/shielding to protect surrounding normal tissues on the XStrahl kilovotage (kV) irradiator, and one advantage of working with subcutaneous allografts/xenografts is that this avoids irradiation of internal organs. We also ensure that the mice are kept warm in the irradiator (max 10-15 mins for the procedure) using a microwaveable heat pad covered with a heat blanket, and recovered in prewarmed cages. Eye gel will also be used to prevent drying of eyes during the procedure. Mice will be monitored by CCTV camera during irradiation and monitored continuously until recovery. Soft food and water will be put in recovery cages to give easy access to food and water for recovering mice.

If a mouse develops a wet ulcer following irradiation (grade 4 or above on University of British Columbia guidelines, see below), they will be immediately humanely killed.

Where mice are held in individual cages for up to 24 hours for faeces collection, we shall avoid using metabolic cages, but rather shall use conventional cages which will be less stressful. Mice will be kept fed and fully hydrated. During the 24 hours of isolation a small amount of nesting material from the home cage will be kept in the isolated cage. This can help with thermoregulation and makes the new surroundings more familiar.

Antibiotics and metabolites will be delivered by a number of routes, using isotonic aqueous vehicles where possible, using the smallest volume that can be accurately and safely administered. Usually, information regarding doses which cause few or no adverse effects

will be available, but failing this, dose setting experiments may be performed. Gavage of antibiotics may require larger volumes than recommended by Workman et al or Morton et al (see below), as we may require up to 200 ul in 3-4 week old mice, some of which may weigh less than 10 g. Where possibly, we will try to use lower volumes if this does not compromise the scientific objectives. Metabolites, including short chain fatty acids and, for example, isoferulic acid, will be given in physiologically relevant doses, not expected to cause adverse effects. Sweetener will be added to drinking water where substances are found to be unpalatable.

Suffering can also be limited by planning radiotherapy to limit dose to surrounding normal tissue, and also humane killing of mice whose small bowel is irradiated within 3-5 days, i.e. before the onset of the gastrointestinal syndrome.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All experiments will be based on Workman *et al* 'Guidelines for the welfare and use of animals in cancer research' *Br J Cancer* 2010; 102:1555-77, including tumour cell inoculation volumes.

Morton DB *et al* Refining procedures for the administration of substances. Report of the BVAAWF/FRAME/RSPCA/UFAW Joint working group on Refinement. *Lab Anim* 2001:1-41 will be used for standard routes of administration or blood sampling unless otherwise stated. If volumes/frequencies are expected to exceed these recommendations, they will be agreed in advance with the Home Office Inspector after taking advice from the NVS.

The University of British Columbia ACC Guideline on rodents with ulcerated subcutaneous tumours will be used to assess skin/tumour ulceration. https://animalcare.ubc.ca/animal-care-committee/sops- policies-and-guidelines/acc-guidelines

PREPARE guidelines will be used in the planning of experiments and ARRIVE guidelines followed subsequently.

We will consult other resources including guidance and publications from the NC3Rs and LASA as appropriate.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will regularly check information on the NC3Rs website and attend local/regional courses and online webinars. We receive information regularly via local meetings and from staff in the animal unit regarding key training events and 3Rs advances.

97. Drug delivery for diseases of the central nervous system

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Brain, Cancer, Parkinson's, Cerebrospinal, Neurodegeneration

Animal types	Life stages
Sheep	adult
Pigs	juvenile, adult
Minipigs	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To assess the potential of delivering drugs to the central nervous system utilising natural fluid drainage pathways and to facilitate the development of the technology needed to achieve this.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Diseases of the central nervous system (CNS) are a major cause of suffering and death. The lack of effective treatments for many disease of the CNS constitutes a largely unmet clinical need. A major obstacle to the treatment of diseases of the CNS is the difficulty in

achieving therapeutic drug concentrations. Most drugs used in the treatment of diseases are prevented from entering the CNS by a protective layer, known as the blood brain barrier, which is a feature of the blood vessels within CNS. As a result, the development of effective treatments for brain tumours and neurogenerative diseases such as dementia, Parkinson's, Alzheimer's and Motor Neuron Disease had been severely limited.

Consequently, there is an urgent need to develop drug delivery systems that can circumvent the limitations imposed by the blood brain barrier in order to address the unmet clinical need of patients.

What outputs do you think you will see at the end of this project?

The primary outputs from this project will be data relevant to assessing the effectiveness and safety of delivering drugs to the central nervous system via the cerebrospinal fluid which surrounds it. The data generated will be used to support applications to translate this approach into the clinical setting and will be published in peer reviewed scientific journals, open access sources and presented at medical conferences.

Who or what will benefit from these outputs, and how?

In the short term, the information gained will be of benefit to scientists, engineers and clinicians working to develop ways of delivering therapeutic concentrations of drugs to the brain.

In the medium term, the work is expected to benefit clinicans by facilitate the translation of a novel delivery system, for use in the treatments of a range of neurological disease including brain tumours and neurodegenerative disorders, into the clinical setting.

In the long term the work is expected to benefit patients suffering from neurological disease.

How will you look to maximise the outputs of this work?

The findings of the study will be disseminated through presentations at scientific conferences and publications in peer reviewed journals, whenever this is not precluded by intellectual property rights.

Species and numbers of animals expected to be used

- Sheep: 50
- Pigs: 30
- Minipigs: 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.

The outlined studies aim to generate the data needed to translate novel drug delivery systems, for the treatment of neurological diseases, into a first in human clinical trial. To this end, the study requires the use of an animal with a central nervous system that is of a similar size and structure to that of humans. Pigs and sheep have been chosen as they meet these requirements and are readily available. Juvenile commercial pigs will be used for short term studies involving a single treatment while for longer term studies, in which drugs may be given repeatedly or continuously via an implanted delivery device, either adult mini-pigs or adult sheep will be used to avoid the risk of the delivery cannula becoming displaced due to the growth of the animal during the study period.

Typically, what will be done to an animal used in your project?

Upon arrival in the unit, the animals will be habituated to humans, by regular close contact and hand feeding them palatable treats. The animals will be trained to voluntarily enter the weighing crate and transport trollies. On the day of surgery, the animals will be moved to the surgical suite and anaesthesia induced while the animal is in the transport trolly, to minimise any associated stress.

Anaesthesia will be maintained using mechanical ventilation while the animal undergoes a surgical procedure replicating that intended for human clinical practice. During the procedure the animal will undergo non-invasive imaging using a CT or MRI scanner. Where necessary, the animal may be fitted temporarily with an external reference frame to enable the accurate insertion of a modified shunt catheter into either: 1) the ventricles of the brain (intracerebroventricular (ICV),or 2) the cisterna magna (intra-cisterna magna (ICM), or 3) the lumbar theca (intrathecal (IT). For long term studies the catheter will be fixed in place and may be attached to an implanted delivery device or external port. Contrast agents or drugs may be delivered and further imaging undertaken. Animals will then either be killed, to enable the brain and/or spinal cord to be harvested for analysis, or allowed to recover from the anaesthetic. Animal permitted to recovered may undergo further non-invasive imaging and/or be given agents via the an external port before being killed by anaesthetic overdose.

What are the expected impacts and/or adverse effects for the animals during your project?

The animals are expected to habituate to human contact and to learn to confidently enter the transport trolly and weighing crate within a few days of arrival. Upon the day of surgery, the animals will be weighed and moved to the surgical suite using the transport trolly. Anaesthesia will be induced while the animal is in the transport trolly to minimise any associated distress. During induction, the animal will experience mild transient pain, caused by the insertion of a hypodermic needle, and mild transient distress as the anaesthetic takes effect. Animal permitted to recover following surgery will experience some post-surgical pain which will be mitigated by the administration of analgesic agents, given under the direction of a specialist veterinary anaesthetist. Post-surgical recovery is expected to be uneventful and all animals are expected to resume normal behaviour within a few hours. The subsequent administration of agents is not expected to have any adverse effects on the animals. At the end of the study period the animal will be anaesthetised and killed by anaesthetic overdose.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Sheep- Moderate (80%) & Non-Recovery (20%)
Pigs- Moderate (80%) & Non-Recovery (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?

In order to translate novel technological developments and treatments into clinical practice, it is essential to demonstrate their safety and efficacy using representative animal models that meet the requirements of the regulators responsible for authorising first in human clinical trials.

Which non-animal alternatives did you consider for use in this project?

Non-animal alternatives are not relevant to this study as only data generated using a representative animal model will suffice to meet the requirements of the regulators responsible for authorising first in human clinical trials.

Why were they not suitable?

Only data generated using representative animal models meets the requirements of the regulators responsible for authorising progression to human clinical trials. Consequently, there is no alternative to the use of animals for the outlined studies.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimated number of animals required for the outlined studies has been determined using data generated during similar work conducted under my previous licence and using the NC3Rs Experimental Design Assist application.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Power calculations were undertaken to determine the experimental group size, using data generated during similar work conducted under my previous licence. The experimental design was verified using the NC3Rs Experimental Design Assist application. Where appropriate, control group data obtained in previous studies will be used to minimise or eliminate the need for specific 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?

Where appropriate, control group data obtained in previous studies will be used to minimise or eliminate the need for specific controls.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The animals used in the outlined studies will undergo a procedure that replicates that intended for used in a medical setting. Suffering will be minimised by habituating the animals to humans, by regular close contact, hand feeding them palatable treats and training them to voluntarily enter the weighing crate and transport trollies used to move them to the surgical suite. Other than the induction of anaesthesia, all procedures will be conducted while the animal is anaesthetised. Any animal allowed to recover following surgery will be given analgesic agents to control pain, under the direction of a specialist veterinary anaesthetist, until no overt signs of pain are detectable. At the end of the study the animals will be humanely killed using an overdose of an anaesthetic agent.

Why can’t you use animals that are less sentient?

To undertake the outlined study, an animal with a brain of similar anatomical size and structure to that of humans is required. Only mammalian species of a moderate size, such as the pig and sheep, are able to meet these criteria.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Upon arrival in the unit, the animals will be habituated to human contact before any procedures are undertaken. Surgery will be performed by a specialist surgeon with extensive experience in the use of animal models to facilitate the translation of new and improved procedures into first in human clinical trials. Surgery will be conducted in a manner that replicates that intended for translation into a clinical setting. Following surgery, the animals permitted to recover will be provided with post-operative pain control, under the guidance of a specialist veterinary anaesthetist, until they show no discernible signs of pain. All animals are expected to resume normal behaviour within a few hours of recovery from anaesthesia and to continue to live normally throughout the study. I am

committed to ensuring that all the procedures undertaken by my group are refined to minimise suffering and that the experiments are designed to use the least number of animals needed to obtain the required data.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The procedures used will closely follow those intended for use in a clinical setting within the NHS and will be conducted using full aseptic precautions in line with LASA guidelines for aseptic surgery.

Studies will be conducted in compliance with GLP standards.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

My institute places a strong emphasis on the promotion of the 3Rs and organises regular event to raise awareness and encourage the dissemination and uptake of 3Rs developments. We are supported by a regional NC3Rs representative, who actively publicises and promotes engagement with the 3Rs. The institution's AWERB committee promote the 3Rs by challenging licensees to demonstrate their full commitment during licence reviews and the NVS and NACWO review all pre-study briefings and raise any 3Rs concerns with the applicant when they arise.

98. Imaging Method Development

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

Imaging, Medicine Discovery, Clinical Translation

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 refine imaging methodologies to optimise pre-existing methods, and also to implement more robust and/or imaging methods used in people in support of the research and development of new medicines.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Non-invasive imaging is a reproducible and powerful tool which has been increasingly used in medicine discovery projects.

Non-invasive imaging methods have been widely used in the company to aid in the development of new medicines by providing information which cannot be gained by traditional methods. Examples of this information include but are not limited to; determining where drugs are distributed in the body, characterising animal models of disease and also helping to determine whether potential new medicines can reduce disease severity in animal models.

Whilst imaging methods which have been used over the last 20 years have provided key information in the testing of potential medicines, new imaging methods are being continually developed by imaging system manufacturers and academic researchers, and these may further improve the existing methods. These improved methods may take several forms, from a simple reduction in scan times, through to fundamentally different methods for how image data is acquired.

These novel or refined methods do not usually transfer directly from vendors or published methods without modifications to make them work appropriately on different imaging systems, so it is critical that in-house refinement and validation of new methods is conducted prior to them being applied to routine studies.

Many imaging studies within medicine discovery are aimed at providing animal data which is relevant to imaging in clinical trials. As such where possible imaging methods used in clinical trials can be applied in earlier animal studies, which could help improve the development process for new medicines.

What outputs do you think you will see at the end of this project?

The outputs from this project will be the routine application of new methods for use on studies conducted under other project licences at this and other establishments.

Specifically, reduction in scan duration directly results in a reduction in the time for animals to be anaesthetised, and hence less burden on the animals. This is particularly important when applied to disease models where the effect of anaesthesia may be greater than on non-diseased animals e.g., if the animals breathing is affected by the disease model being investigated.

Routine application of improved or novel imaging biomarkers, (naturally occurring molecules that can help us understand disease), on other project licences at this establishment will better support efforts to reduce late-stage attrition, (e.g., medicines failing in the clinic), by adding greater predictivity to pre- clinical studies.

Who or what will benefit from these outputs, and how?

Application of novel optimised imaging methods will directly benefit projects by providing either better quality data, or by providing biological characterisations which were not previously possible, but which will aid in appropriate progression of potential new medicines.

The welfare of animals may also benefit by potentially reducing the burden of anaesthesia by either reducing the required anaesthesia times, or by reducing the number of imaging, and thereby, anaesthesia sessions required.

How will you look to maximise the outputs of this work?

The company has adopted the principles of robust study design that incorporate the standards of the PREPARE and ARRIVE guidelines. Peer review (including statistical review) during the experimental design phase will contribute to achieving the high experimental quality required for publication. Where work is considered to be 'pre-competitive', (e.g., method development, model validation) i.e., it does not contain information that is subject to intellectual property constraints, it will be strongly considered for publication, as occurred with experiments carried out under prior licence authorities, and as part of consortia such as the EU Innovative Medicines Initiative (2010-1015). The company supports the premise that publication of unsuccessful approaches ('negative data') is a valuable scientific output from properly conducted research and this would not be excluded publication.

Data generated for this project is stored in a searchable, data integrity compliant, backed up company database so that it remains accessible to other company researchers. Therefore, data will be recoverable in the future even after likely project and personnel changes and will be a valuable resource to reduce the need to repeat and re-establish competency in a field of research.

Species and numbers of animals expected to be used

- Mice: 500
- 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.

Work under this licence will be carried out using either rats or mice. To date, whilst there is an increasing application of non-animal alternatives, animal models can still not be fully replaced in all pre-clinical research; they are required to both answer basic research questions and to assess the activity of potential new medicines in complex inter-connected system environments. Although there are known differences between rodent and humans, the similarities in systems and published
knowledge of underlying immunology facilitates the use of rodents, and mice in particular as invaluable in the discovery of new medicines, and as such the mouse remains the most commonly used laboratory animal in research.

Only adult animals will be used, as the majority of our work will be to better understand the involvement of a mature immune system. For rodents this would typically require individuals of at least 8 weeks of age.

Typically, what will be done to an animal used in your project?

Naïve animals will be anaesthetised and placed in imaging systems for assessment. Animals may receive agents prior to or after anaesthetic via injection routes e.g., into a vein, which will improve image contrast which will allow specific organs or body processes to be visible. Depending on the objectives of specific studies, animals may be either recovered for repeat imaging sessions or euthanased. For optical imaging, removal of fur by shaving and/or application of depilation cream may be required.

All animals will be euthanased at the end of the licence protocol.

What are the expected impacts and/or adverse effects for the animals during your project?

Dependent on strain of animal it is possible that a mild skin rash could develop after shaving or hair removal.

Some animals will experience transient bodyweight loss following anaesthesia and/or administration of imaging contrast agent.

Some animals will experience mild, transient discomfort associated with the injection to administer imaging contrast agents or for blood sampling. The agents in themselves will not have an impact or cause adverse effects.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Due to shaving cuts or skin rash associated with depilation a maximal severity of mild is expected in a low incidence of animals (<5%)

It is expected that transient bodyweight loss may be expected in a low incidence of animals (<5%) following anaesthesia and/or administration of imaging contrast agent.

It is expected mild, transient discomfort associated with the injection to administer imaging contrast agents and/or blood sampling will be experienced by ~40% of animals.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.
Why do you need to use animals to achieve the aim of your project?

Application of methods optimised under this project are designed specifically to be applied to animal experiments on other project licences. As such the use of animals is necessary to provide the improvement in existing animal imaging methods.

Which non-animal alternatives did you consider for use in this project?

As the ultimate application of the methods developed on this license are solely for animal studies, there are no absolute replacements for using animals. However, prior to an in vivo experiment, where appropriate, initial work will use surrogates such as imaging phantoms (e.g., tubes of copper sulphate solution), after which further method development may be conducted on isolated organs and/or cadavers before ultimately imaging live animals.

Why were they not suitable?

Alternatives do not replicate the whole intact living system especially when looking to address specific requirements to gather high quality images that are not affected by respiratory or cardiac motion i.e., developing motion-insensitive image acquisition methods. The methods to be used, developed and refined under this licence are specifically for use in animal studies on other project licences investigating new medicines to treat disease.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Based on the number of planned imaging method development investigations over the next 12 months, with the majority of current work being focused on mice, an estimate of 100 mice and 40 rats per year will be required during the 5 year authority of this licence.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Before performing any work under this project, non-animal experiments will be utilised to conduct a proof of concept for any novel imaging method. This is usually achieved by use of imaging “phantoms” – which often take the form of a simple device replicating specific aspects of what will be imaged. For example, bone-mimicking phantoms may be used in Computed Tomography (CT) methods; copper sulphate solution is the phantom of preference for mimicking the water content of an animal in MRI; solutions of fluorescent agents, or Light Emitting Diode systems are often used for the initial optical imaging method workup.

These phantom experiments will be utilised until no further useful information can be gleaned from them, whereupon cadaver experiments will usually be conducted prior to conducting the first in vivo experiment.

All experimental work is planned with the input of biostatisticians to ensure that experiments provide high quality data using the minimum number of animals. Experimental group size will either be based on existing data, or small trial (pilot) studies will be carried out to establish the variation of the imaging endpoint being investigated. Understanding the variation and what constitutes a meaningful difference between groups will allow a statistician to calculate group sizes that are used to ensure that statistically meaningful comparisons can be made e.g., between pre-existing and refined imaging endpoints.

These design principles aim to reduce the possibility of experiments not generating decision making data, potentially resulting in repeating work and, hence using more animals. In addition to statistical support, all studies conducted under this licence will undergo internal peer review in order to ensure that all aspects of experimental design are scientifically suitable for the study being proposed.

Robust study design measures, (e.g., randomisation, blinding, power calculations,), will maximise the likelihood of generating non-biased experimental results, and limit the number of animals required to optimise new imaging methodologies.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Where improving an existing imaging method on an in vivo study, the original and improved methods will usually both be acquired within the same animal allowing for a direct comparison between the methods to be made. For example, the comparison could be a simple reduction in scan acquisition time, or increased sensitivity of the imaging method thus allowing for a direct comparison between new and legacy methods to be made. Depending on the novel imaging method being developed, pilot studies may be required prior to head-to-head comparison with existing methods.

Where novel methods are to be developed and there is no pre-existing imaging method with which to compare e.g., immune cell imaging, then other appropriate approaches e.g., histology, MALDI can be utilised to validate the imaging method.

Blood samples may be taken to aid in the development and characterisation of imaging methods, particularly where imaging contrast agents have been used as blood sampling can aid in disconnecting circulating imaging signal from tissue-based signal.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Only naïve animals will be used under this project licence, with application of methods developed under this licence to be applied in disease models on other licences.

The 4 methods to be used in this project are anaesthesia, administration of imaging agents, blood sampling and fur removal by shaving/depilation.

As the primary potential harm of non-invasive imaging is the anaesthesia, which is necessary for the acquisition of scans, this potential harm will be minimised by application of best practices for anaesthesia such as minimising the duration of anaesthesia, along with supportive measures such as monitoring and maintaining animals' temperature and e.g. heart rate and respiration whilst also factoring in potential impact of anaesthesia duration and frequency in repeat imaging studies.

Recommended and best practice will be applied for all procedures before, during and post-imaging e.g., anaesthesia, dosing, blood sampling, post imaging care including consideration of potential impact of repeat imaging and anaesthesia.

Why can't you use animals that are less sentient?

The methods developed in this project will be applied to in vivo work covered by other project licences. As such, this work must be conducted in the same species, and often strain, as will be used in these licences at this establishment.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Minimising welfare issues will be addressed by understanding the requirements, collaborative decision making/planning and using trained staff with experience at recognising potential issues should they occur. Close working relationships will ensure the study is required and that the study design appropriately meets the objectives.

As the primary potential harm of non-invasive imaging is the anaesthesia, which is necessary for the acquisition of scans, this potential harm will be minimised by application of best practices for anaesthesia such as; minimising the duration of anaesthesia, supportive measures such as maintaining animals' temperature, monitoring e.g. heart rate and respiration and post-imaging care such as placing animals in a warm environment until full recovery, whilst also considering the potential impact of anaesthesia duration and frequency in repeat imaging studies. Additionally, where compatible with the objectives of the study, non-recovery anaesthesia will be used.
Where blood samples are taken, micro sampling techniques will be used whenever possible, to make sure the minimum sample (e.g., microliters) volume is obtained using the least invasive techniques.

Where possible, combining procedures such as shaving and blood sampling under a single anaesthetic session will be utilised.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The published principles and philosophies behind the PREPARE (2018) and ARRIVE (2020) guidelines have been incorporated into the sponsoring companies internal project planning standards of care and standard operating procedures. All work carried out under authority of this licence will undergo assessment of the design during study planning as part of a robust study design process that is based on those guidelines. Facilities and processes are audited by independent bodies such as AAALAC which has published guidelines and procedures to ensure work is carried out to high ethical and humane standards.

Maximum dose volumes and dosing frequencies and durations will be applied e.g., as described in ' Diehl KH et al. (2001). A good practice guide to the administration of substances and removal of blood, including routes and volumes. Journal of Applied Toxicology 21(1): 15-23, and Morton DB et al. (1993). Removal of blood from laboratory mammals and birds. Laboratory Animals 27(1): 1-22.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay informed about advances in the 3Rs through regular referral to the Norecopa and NC3Rs websites, imaging conferences, as well as other published literature.

Furthermore, the establishment Named Information Officer (NIO) facilitates the dissemination of information via newsletters in relation to any such advances. In accordance with any updates, we will review and revise the protocols within this licence to ensure they have been adequately considered, and where applicable, applied.

Awareness of imaging specific refinements will be maintained e.g., by review of published literature and attendance at relevant conferences.

99. Investigating the genetic causes of cardiovascular 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

Atherosclerosis, Genetics, Aortic valve, Cardiovascular disease

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The primary aim of this study is to understand the mechanisms by which genes that associated with increased risk of cardiovascular disease (CVD) contribute to disease development and progression. A secondary aim is to progress towards new treatments by targeting the key genes and pathways identified through our human genetic studies.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

CVDs are responsible for more than 20 million deaths each year. Treatment is primarily preventative and aimed at counteracting modifiable lifestyle risk factors through lowering cholesterol and blood pressure, stopping smoking, maintaining a healthy weight and

regular exercise. However, even with these preventative measures, the number of yearly deaths caused by CVD is rising. To combat this, we need to better understand the causes and processes that lead to someone developing a CVD and use this knowledge to develop new treatments. A person's family history is another key risk factor for developing a CVD is their family history. Differences in a person's DNA can double or triple their risk of disease. These genetic changes are passed on from parents to their children and shared within families. Over the past 15 years we have made substantial progress in identifying the genetic changes that explain the genetic risk of CVDs. In most cases, however, we do not understand why these genetic changes contribute to the development of disease. The work here will contribute to our understanding of the key genes and molecules involved in the disease process, leading to a better understanding of disease development and progression, genetic screening to identify those individuals most at risk and the development of more targeted and effective treatments, ultimately improving patient outcomes.

What outputs do you think you will see at the end of this project?

The primary output of this research will be improved knowledge of CVD pathogenesis communicated via the publication of scientific manuscripts and presentations to scientists and patient groups. A secondary output will be the confirmation of specific genetic variants and genes as causal for CVDs which will be reported in the literature, recorded in publicly available clinical databases and communicated with our clinical colleagues and genetic counsellors. A final output is the identification of new candidate therapies as measured by the progression of our findings towards in-human studies.

Who or what will benefit from these outputs, and how?

Most of our work relates to advancing our understanding of disease pathogenesis and will benefit the scientific community (short-term). Our research goals also aim to provide benefit for patients.

Validating genetic changes as causal for disease can impact patient care by enabling more effective genetic counselling, screening and disease prevention. Benefits are expected in the mid-term for patients where rare genetic variants have a strong contribution to disease risk. These benefits will likely be for a smaller number of patients and begin with the individual families in our care. Long-term, with improved understanding of the risk conferred through the smaller effects of more common genetic variants, we aim to better identify those individuals who might benefit from earlier preventative treatments due to their higher genetic risk of disease. Finally, within the course of this work we aim to identify new therapeutic targets and progress these towards in-human studies providing new treatment options not currently addressed by other medication for CVDs (long-term).

How will you look to maximise the outputs of this work?

Findings will be made available through open access publication in peer-reviewed journals and presentations at meetings and conferences. All genetically altered animals will be made available to other researchers

Species and numbers of animals expected to be used

- Mice: 1850

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Most cardiovascular diseases are caused by genetic and environmental changes and involve the interaction of multiple different tissues and cell-types. As such, some experiments require animal models to fully understand and decipher disease pathogenesis. This projects will utilise mice as these are an established experimental model for the cardiovascular diseases and pathogenic processes we investigate and the genetically altered animals required for this research are generally already available. Atherosclerosis, which is the cause of coronary heart disease in most people, occurs over a long period-of-time. These experiments therefore involve adult mice with genetic and dietary changes utilised to induce and model disease. Experiments usually involve the investigation of disease progression in adult mice over several weeks. Spontaneous coronary artery dissection is a less frequent cause of coronary heart disease which predominantly affects younger women. While pathogenesis is not fully understood, it is believed to involve the weakening of vessel elasticity and compliance. Experiments therefore require analysis and measurement of vessel function in adult animals. Bicuspid aortic valve is a common congenital heart defect and usually benign until later life. Most experiments will require assessment of phenotype in adults, however since bicuspid valve is a developmental disorder a proportion of embryos will also be required. Most of our work, across all projects, predominantly involves human studies, in silico analysis and human cellular models. We only progress to mouse models for a small number of specific analyses where our non-animal work is not sufficient to gain the knowledge required.

Typically, what will be done to an animal used in your project?

Most mice in this study will be used for breeding to generate experimental groups with the desired genotypes. Most of these animals will not be subject to any other procedure with investigations involving the collection of tissue for post-mortem phenotyping, ex vivo techniques, in vitro cellular studies, histology and gene and protein expression analysis.

Some mice will undergo at least one standard technique such as an injection (e.g., to administer a substance to induce a mutation or a compound that modulates disease) and/or blood sampling and/or general anaesthesia. Anaesthesia may be non-recovery or used for restraint purposes (e.g., for imaging).

Our investigation of atherosclerosis means that most mice within this category will receive a high fat diet, usually for 12-weeks.

Some mice may be studied over long periods (no more than 12 months) to allow the development of pathologies.

What are the expected impacts and/or adverse effects for the animals during your project?

Most mice will be used for breeding and are not expected to experience adverse effects. Of those that do, the exact effects are difficult to predict but are not anticipated to exceed mild effects such as slightly reduced food consumption and weight gain. A smaller number of

mice will experience at least one standard technique such as an injection and/or blood sampling and/or general anaesthesia.
Anaesthesia may be non-recovery or used for restraint purposes e.g., for imaging. Most mice in this category will also receive a high fat diet and although they gain weight it is not to the extent that if affects normal behaviour. Most mice are anticipated to experience a mild severity, but as the cardiovascular system is under investigation, mice experiencing a moderate severity cannot be completely ruled out. At all stages, mice are monitored routinely and those causing any concern (or if they could potentially become ill for example if they have just received a procedure) are monitored more closely. Analgesia is used routinely under advice from the vet or welfare officer regarding the selection of an appropriate agent that will not affect scientific outcome.

Expected severity categories and the 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, >95%, are not expected to exceed mild effects. No more than 5% are expected to show moderate clinical signs.

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?

Cardiovascular disease is a whole-body disease involving multiple organs and different cell-types and so the use of animals cannot be completely replaced. Where possible, non-protected animal alternatives are used.

Which non-animal alternatives did you consider for use in this project?

Most of our work does not involve animals. The majority of our work involves the discovery of the genetic causes of cardiovascular disease. We have several large patient cohorts and contribute to international cohorts in our genetic analyses. We recall patients for in-human studies (e.g., imaging) and collect materials (e.g., cells) for functional validation experiments. We also host the largest collection of human vascular cells in the world. We always perform substantial in vitro analyses and only proceed to in vivo approaches when completely required.

Why were they not suitable?

Non-animal models are suitable for most of our experiments. However, some lines of investigation do require experimental studies in to the consequence of genetic or

molecular changes on cells and tissues within the body where animal free models are not 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?

Most animals will be required for breeding to generate the genotypes required for our experimental studies. Numbers are based on our past-experience, an estimate of the number of genes we intend to investigate. Sample size for experimental groups are calculated in collaboration with statisticians within our research group so that the minimum number of mice are used to reach our project objectives.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Our approach is based on our previous experience, related literature and input from our collaborators. Our core group includes senior statisticians who provide input to experimental design and support our analyses. We will utilise newer experimental models so that fewer animals are required for breeding and design ex vivo experiments so that multiple lines of investigation can be carried out from a minimum 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?

Our experiments are informed from data generated through large-scale human studies and extensive in vitro analyses. We consult with the experienced technical staff within our facility so that our breeding strategies are efficient, perform pilot studies so that experiments are designed to produce meaningful data through the of a minimum number of animals.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Our studies on cardiovascular disease will employ mice as they are an established and widely-used model in this field. Genetically altered animal models for most of our genes of

interest already exist, allowing us to conduct our experiments efficiently. In addition, extensive phenotypic data is available for these models, which enables us to design experiments that minimize the potential for adverse effects related to genotype.
We expect that most animals used in our studies will only develop sub-clinical phenotypes, and will exhibit no more than mild effects, such as reduced food consumption and weight gain. However, a small number of mice may experience moderate effects due to the disease. To minimize any negative impact on the animals, all mice will be routinely monitored throughout the study, and those causing any concern will be monitored more closely. Analgesia will be provided where needed.

Why can’t you use animals that are less sentient?

Less sentient models do not reflect the disease state of mammals. Most of our experimental data is generated following schedule 1 killing of genetically altered mice that do not experience any other procedure. The initiation and progression of atherosclerosis is extremely complex. Mice do not normally develop disease without genetic manipulation or the administration of substances and alteration in diet. Disease progression is mediated through several risk factors such as plasma lipid levels and blood pressure, which need to be recorded so that the processes underlying disease can be understood.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Throughout this project, we will refine our methodology based on our results, input from collaborators, relevant publications and the guidelines issued by the Home Office and NC3Rs.

Previous refinements include the introduction of handling tubes into the home cage at least a day before performing non-invasive blood pressure measurements, pre-warming the room so as the mice are handled in tubes on a warming plate for as little time as possible, and using a reduced feeding technique when taking fasting blood measurements so as mice are fasted for around 6 hours rather than overnight. For all procedures, animals will be monitored closely for signs of suffering. All in vivo approaches will be based upon causing the minimal amount of suffering to achieve the required objectives and approaches causing adverse effects will be reassessed to determine whether a different approach would be more suitable.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We are committed to conducting our experiments in the most refined way possible, in line with the guidelines provided by the NC3Rs. To stay up to date with the latest developments and best practices in the field, we will subscribe to the electronic version of the NC3Rs' monthly newsletter.

In addition to the NC3Rs, we recognize the important work of the Nuffield Council on Bioethics in promoting animal welfare and the refinement of animal experiments. We will consult their published work as needed to ensure that our research adheres to the highest ethical standards.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our approach to ensuring the responsible use of animals in research will be guided by the NC3Rs and other relevant sources of information. We plan to attend symposia and conferences on 3Rs, and stay informed about the latest research and best practices by reviewing published literature. The NC3Rs website is a particularly valuable resource for guidance on topics such as animal welfare, experimental design, and animal housing and husbandry.

In addition, we recognize the importance of seeking advice from experts in the field, and will seek guidance from the 3Rs experts at the NC3Rs.

We are committed to implementing the latest advances in 3Rs in collaboration with our colleagues. This will help ensure that we are using animals in research in a way that is both ethical and scientifically sound.

100. Investigating the inflammatory processes that support formation of atherosclerosis in arterial disease

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Cardiovascular disease, Inflammation, Risk factors, Therapies

Animal types	Life stages
Mice	neonate, juvenile, adult, pregnant, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We aim to describe the role of inflammation and thrombo-inflammation (interactions between the blood clotting system and the immune system) in the development of atherosclerosis. This is a disease where fatty deposits form plaques on teh artery wall, which upon rupture can cause heart attacks and strokes. We will determine whether new therapeutic options are available based on novel immune regulatory agents and pathways which intervene in inflammation (and thrombo-inflammation) driven disease of the artery wall which might reduce the risk of heart attack and stroke.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

World Health Organisation statistics show that an estimated 17.9 million people died from cardiovascular diseases (CVDs) in 2019, representing 32% of all global deaths. Of these,

85% were attributed to heart attacks and strokes. Both of these CVDs are caused by atherosclerosis, which is a chronic inflammatory disease of the artery wall which leads to the formation of atherosclerotic plaques which can cause blood clots that block the arteries in the heart (heart attack) or brain (stroke). There is currently no cure for atherosclerosis and a major reason for the lack of appropriate medicines is our poor understanding of the molecules and cells that initiate and support inflammation in the artery wall during plaque formation.

What outputs do you think you will see at the end of this project?

We will generate new information on the role of inflammation in cardiovascular disease. These studies will be published in peer reviewed journals. We are also working on new anti-inflammatory pathways and anticipate that new pharmaceutical targets will be identified for development of new medicines.

Who or what will benefit from these outputs, and how?

In the short term we will generate new information that will benefit the field of cardiovascular research, enabling other scientists to use this knowledge in the design and execution of their experiments. In the medium term, we aim to identify new pathways that regulate inflammation which will be used as targets for development of new medicines. In the longer term (beyond the lifetime of this PPL) we hope to deliver new treatment options to the clinic, so that patients with cardiovascular disease benefit in terms of their health.

How will you look to maximise the outputs of this work?

The work will require collaboration between a number of laboratories nationally and internationally who all have discreet expertise in analysing the experiments to be conducted. This means that material (e.g. tissues and blood) will be subject to a wide range of scrutiny by highly specialised laboratories, thereby maximising the quality of data generated. The study data will be published in peer reviewed, free access journals, so that it is available to the widest constituency of interested readers. New findings will be presented to the scientific community at national and international symposia regularly and in a timely fashion. Very large data sets of gene or protein expression profiles will be archived on appropriate and discipline specific data bases, which are free to access for other scientific groups.

Species and numbers of animals expected to be used

- Mice: 3100

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Adult mice are used to model the development of cardiovascular diseases. These are necessary as the chronic process of disease development is known to involve numerous organ systems and tissues such as the immune system, metabolic system, and cardiovascular system. These cannot be studied in isolation and require the integrated

activity that occurs in the whole animal. Mice represent the most tractable mammalian species from the point of view of concordance in physiology (including the immune system), allied with rapidity of disease development, and availability of genetic tools. The disease process although much accelerated compared to humans does bear a striking similarity to disease in adult humans, but adult mice are required as the process of disease development still takes several months.

Typically, what will be done to an animal used in your project?

Mice, including those that are genetically modified, will be subject to dietary variation for up to 24 weeks (e.g. high fat diet) to induce high cholesterol in the blood, which is necessary to induce formation of atherosclerotic plaques.

They may also receive agents such as inhibitors of enzymes or blockers of receptor function, that regulate the trafficking of immune cells from the blood to the artery wall, and/or can regulate the activity of the blood clotting system over the duration of an experiment (usually 12 weeks; maximum of 3 a week) or if more chromic administration is required, then a subcutaneous osmotic minipump will instead be implanted, lasting up to 6 weeks and replaced a maximum of once.

At the end of an experiment a minority of animals may be used to observe the movement of blood cells in blood vessels and tissues whilst they are under none-recovery anaesthesia.

What are the expected impacts and/or adverse effects for the animals during your project?

High fat feeding is associated with weight gain and additional grooming, as their coats become greasy, which may result in the occasional animal with skin sores. In combination with genetic alteration, high fat feeding makes animals prone to CVD but they do not suffer heart attack and stroke upon development of disease and the process is otherwise benign.

Injections may cause localised transient pain.

Surgery for minipump insertion is associated with short term pain which is managed by analgesia.

Induction of inflammation by injection of inflammatory mediators can result in a short period (<24h) of discomfort during the induction period from which animals recover rapidly.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

75% Mild
25% 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?

As with many chronic inflammatory diseases, cardiovascular disease is a systemic disease involving dysfunction of numerous organs and systems. For example, the immune system, the metabolic system, and the cardiovascular system all make major contributions to the development of atherosclerosis. Moreover, disease development is driven by multiple risk factors, such as high circulating cholesterol, high blood pressure, obesity etc., which are integrated systemically within the body to lead to vascular dysfunction and chronic inflammation. This leads to the recruitment of white blood cells (inflammatory immune cells) from the blood to the artery wall, as well as proliferation of local cells (smooth muscle cells) and over-production of fibrotic molecules such as collagen. These processes all lead to formation of an atherosclerotic plaque. Such complex and long-term interactions between organ systems cannot be modelled or studied in vitro. Neither can we study the process in patients, as in humans it may take 40 years of symptom free disease development before unpredictable acute disease (such as heart attack and stroke) is evident.

Which non-animal alternatives did you consider for use in this project?

We already utilise numerous in vitro models of inflammation, some of great sophistication, to identify pathways of inflammation that may be relevant to cardiovascular disease. These involve the use of cultured vascular cells (e.g. from the veins of umbilical cords) and isolation of immune cells from donated human blood. We have also pioneered advanced multi-cellular models using tissue engineering principals to rebuild aspects of the diseased cardiovascular system. We also have access to patient material from clinic. This includes atherosclerotic plaques removed by surgery and blood from patients. Together these resources allow us to conduct detailed analysis on inflammatory pathways in CVD and identify new targets for the development of therapeutic agents.

Why were they not suitable?

In vitro models, even multicellular coculture models of the blood vessel wall, cannot recapitulate the systemic interactions of multiple organs and systems and risk factors which underlie the process of chronic cardiovascular inflammation. Integration of all these simultaneously over protracted periods (weeks-months) can only be achieved in vivo. The use of patient material can be useful for verifying the presence of immune cells and molecules in established human disease. However, by definition, patients in cardiovascular clinics have advanced disease that has been developing for 40 years or more and we cannot extract information about the early phases of disease initiation ad development form this source of biological material.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have extensive experience in the use of the models to be used which are well established and have been utilised internationally for nearly 30 years. Thus, using data from previously published studies, and from our own work, we can make accurate estimates of the number of animals to utilise in an experiment. This is followed by the use of specific mathematical calculations based upon these studies and the likelihood of our interventions producing positive results, to estimate the number of animals we will use in our study.

For all experimentation, the lowest possible number of animals will be tested whilst ensuring that the experimental result is robust.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have used statistical analysis to calculate the minimum number of animals necessary for this project.

We will continue to use the NC3R's experimental design tool to aid experimental design and consult trained statisticians before using any new protocols.

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 used statistical analysis to calculate the minimum number of animals necessary for each experiment within this project.

New interventions will first be tested for efficacy using in vitro models prior to use in vivo. Where new routes of administration or new interventions are being examined, pilot studies will first be established in 2-3 mice prior to full experiments. Subsequently these pilot data will used in the specific mathematical calculations described above to ensure that we use the minimum number of animals needed to obtain statistically significant results.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice will be used in models of diet induced inflammation leading to atherosclerosis. The animals, although developing physical disease that closely models that seen in patients, do not suffer acute forms of symptomatic disease, such as heart attack and stroke. Larger model species, such as rabbits, do acquire these symptomatic aspects of arterial disease. Moreover, apart from being obese the mice are otherwise outwardly normal. These are the preferred model of choice world-wide for in vivo studies of this nature because the immune and inflammatory system are very similar in mice and humans.
Moreover, by modifying a single gene (Apo-protein E [ApoE] or low density lipoprotein receptor [LDLR]) mice develop high cholesterol and atherosclerosis spontaneously. The levels of blood cholesterol and disease development can be accelerated by the provision of a high fat diet. This means that animals can be maintained for relatively short periods (e.g. 12 weeks) to develop complex disease. This also means that any interventions (e.g. surgery or injection of immune mediators) need only occur for these short periods.

Why can't you use animals that are less sentient?

Less sentient animals (below the sub-phylum vertebrata, i.e. without backbones) do not possess the same immune systems as humans. Moreover, other vertebrate species (animals with backbones) below the class Mammalia (e.g. fish), do not have equivalent cardiovascular systems to humans. Thus, small rodents are the lowest mammals that can be used to recapitulate the human immune systems and its interactions systemically with cardiovascular system. the use of embryos or terminally anaesthetised animals is not appropriate due to the time required to develop the disease.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Procedures will be refined in line with published scientific developments in the field. In addition, we will review in house data after each series of experiments to determine whether refinements in procedure can be made to minimise welfare costs. Outcomes from in vitro studies may reveal opportunities to look at disease process in shorter time frames. The mode of substance administration will be chosen to cause the least harm and distress to the animal. For example, the use of osmotic minipumps for chronic delivery of agents removes the necessity of multiple weekly injections. Any new substances or route of administration will be tested in a small pilot study and the mice monitored daily for signs of distress. Refinements in husbandry, such as providing food on the floor of housing cages, may alleviate deterioration of fur and skin by avoiding irritation after contact with greasy food

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Animal welfare is a key consideration in all of our protocols, and we will be guided by our NACWOs and NVS in always ensuring that we are using best practice and the most refined techniques. All staff involved in animal experiments will review the literature on animal welfare provided by the local AWERB. Following every experiment and regularly during group meetings we will review our procedures from a welfare standpoint to identify any potential for refinement. We will also follow the published literature on atherosclerosis models for the latest refinements (e.g. https://www.ahajournals.org/doi/epub/10.1161/CIRCRESAHA.122.320263).

The arrive guidelines (Arrive guidelines 2.0 [https://arriveguidelines.org] will be used to ensure that planning, conduct and reporting of studies makes the maximum contribution to the published literature and furthers understanding of the disease process.

We will follow the LASA guidelines Guiding Principles for Preparing for and Undertaking Aseptic Surgery (www.lasa.co.uk/wp-content/uploads/2018/05/Aseptic-Surgery.pdf) and by the Research Animal Training website on Minimum Standards for Aseptic Surgery (www.procedureswithcare.org.uk/ASMS2012.pdf) when undertaking aseptic surgery and providing analgesia.

LASA guidelines on administration of substances will be used to avoid undue distress by selecting the most appropriate route of administration and selecting doses and regimen of delivery that avoid toxicity (https://researchanimaltraining.com/wp-content/uploads/2021/05/lasa_administration.pdf).

PREPARE guidelines will be used during the planning of each study and to ensure timely application of the principals of the 3R's thereby ensuring the validity of the research (https://norecopa.no/prepare/comparison-with-arrive/).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will continue to engage with Establishment efforts to promote the 3Rs and workshops; and receive the NC3Rs newsletter.

101. Mechanisms underlying obesity and ageing associated metabolic disorders

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Type 2 Diabetes, Alzheimer's Disease, Cardiovascular Disease, Type 1 Diabetes, Ageing

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?

The overall objective of our research is to understand why and how obesity, poor nutrition and ageing lead to the development of insulin resistance, type 2 diabetes, cardiovascular disease and neurodegenerative disorders, such as Alzheimer’s disease, and whether we can stop the development of these diseases or reverse it by manipulating the composition of the diet or proteins that are expressed during the development of these. Our aim is to either to prevent, postpone or ameliorate side effects associated with the development of these diseases.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could

be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

In 2023, there are 11 million people aged over 65 in England alone. This is projected to increase by 10% in the next five years and by 32% by 2043 (1.1 and 3.5 million people, respectively). The population aged 85+, the age group most likely to need health and care services, is also projected to rise rapidly, increasing by 8.2% in the next five years and by 62.7% by 2043 (source: Age UK).

However, this increased life expectancy has not been accompanied by a comparable increase in healthspan (the period of life free from age-associated disease). Consequently, by living longer a greater proportion of us will experience age-related diseases (e.g. cardiovascular disease, dementia, type 2 diabetes, sarcopenia), many of which can occur simultaneously or sequentially.

In addition, number of people who are overweight or obese has increased dramatically over the past several decades; e.g. in 2021-2022, 63.8% of adults aged 18 years and over in England, and 67% of adults in Scotland, were estimated to be overweight or living with obesity (source: Gov UK; Obesity Action Scotland). Ageing and obesity are two major factors for development of cardiometabolic disorders. For example, diabetes and diabetes complications account for 10% of total NHS budget (costing £1.5 million every hour).

Diabetic foot ulceration (DFU) is a common complication that occurs in 15-20% of diabetic patients and often requires prolonged hospitalizations and major amputations for its management at an estimated cost of £935 million to the NHS.

Thus, development of a more effective treatment for chronic diabetic wounds is imperative.

By understanding the molecular mechanisms behind the development of age- and/or nutrition and obesity-induced cardiometabolic disorders, we can find novel strategies to combat age- and nutrition/obesity-induced diseases such as diabetes, cardiovascular disease or Alzheimer's.
Considering that we have an ever increasing ageing population and a rapid rise in obesity levels, with poor nutritional intake, across the developed world, understanding these is of utmost importance.

What outputs do you think you will see at the end of this project?

The projects and experiments performed in this licence aim to deliver immediate new knowledge on how ageing and obesity contribute towards development of metabolic diseases by understanding the molecular "switches" that these trigger in the body.

This new knowledge will provide essential new information on the role of ageing, poor nutrition and obesity to health and disease and in particular metabolic diseases such as diabetes, heart disease and dementia. We aim to uncover, both short term and longer term, new treatments for these, both nutritional and pharmacological. The data generated will subsequently lead to publications and presentation to the scientific community both national and international. There will also be significant patient and public involvement and engagement to disseminate research findings to a lay audiences and receive feedback on major complications that patients worry about.

Who or what will benefit from these outputs, and how?

In the short-term, we will be able to address key emerging questions in basic/discovery sciences with potential for health benefit, such as for example, impact of vegan-style diets on cognitive function and diabetes, or emerging promising drugs that we could test immediately. In particular, we focus on complications of diseases that patients suffer from and or which there is currently very few treatment options, eg. Diabetic foot ulcers. These account for around 20% of all limb amputations and are a major complication of diabetes. Using pre-clinical models, such as mice, we can test emerging therapies with the aim of alleviating the progression of the disease faster. This will then also feed into our longer-term strategy of treating a myriad of metabolic diseases.

The identification and development of new (or repurposed) nutritional approaches and/or therapeutics with increased efficacy, reduced side effects and the potential to reverse disease (as opposed to merely treat symptoms) will, overall, lead to improved patient health and a reduced cost and burden on the NHS. Considering that obesity is a major risk factor for development of not just metabolic diseases such as diabetes, heart disease and dementias, but also cancer and other illnesses, research outcomes of this project could have a potential impact on patients and families of those affected by a range of underlying diseases. This would be longer term however as discoveries made in basic sciences often take decades to materialise into new medicines; however, this research is essential as we can often find ways of "repurposing" drugs already available for other diseases that we may potentially use for treatment of metabolic diseases.

How will you look to maximise the outputs of this work?

All work will be presented at national and international conferences and disseminated to the public during outreach and institute open days. In addition, all work will be published in peer reviewed journals. All work will be hypothesis driven and published regardless if confirmed or refuted.

Species and numbers of animals expected to be used

- Mice: 10,650

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice share ~85% homology of their protein coding regions with humans and are the least sentient model that effectively recapitulates the human condition. In addition, disease models of metabolic health, cardiovascular disease and inflammation have been well established in mice to be directly relevant to the human condition. Lower organisms do not have the complexity to adequately model higher complex behaviours or signalling networks.

Experiments performed in this license will use C57BL/6 mice or genetically altered (GA) mouse models mostly on a C57BL/6 background. In addition, there are multiple

genetically manipulated Cre lines commercially available that can be used in these studies. In some cases these may not be on the C57BL/6 background in which case we will attempt to backcross those lines to the C57BL/6 background and/or also investigate how a different strain may relate to the most commonly used C57BL/6. We will generally use mice from 8-10 weeks of age for our experiments and, for high fat/high cholesterol studies, animals will be kept on this diet for approximately 4-5 months. In some cases, since we concentrate on ageing and how ageing affects different genes and disease progression, we will also perform studies in aged colonies. Some colonies will represent models of disease such as for example Tg4510 (Alzheimer's Disease model), Ldlr and ApoE knockout mice (atherosclerosis models), BSCL2 (lipodystrophy model). These are necessary to understand the underlying mechanism(s) of these devastating diseases in humans and if our interventions can help alleviate or even prevent the disease from occurring.

Typically, what will be done to an animal used in your project?

We will use a wide range of techniques to address specific disease questions. For example in an experiment assessing metabolic health of animals and how treatments may affect outcomes, we may follow some of these steps:

Metabolic health and cardiovascular disease. Mice fed a high fat/high cholesterol diet (to mimic "Western style diets) have increased weight gain, develop type 2 diabetes and, in genetically modified animals (eg. ApoE-/-, LDLR-/-), have accelerated atherosclerotic plaque formation (leading to heart disease). We generally begin our experiments when mice are 8-10 weeks of age and are fed either chow (normal balanced diet) or high fat/high cholesterol diet for a up to 20 weeks. During these experiments, mice be assessed for fat and lean mass changes using a Magnetic Resonance Scanner. Mice are awake and free moving and individually placed in a tube and inserted inside the machine.

The procedure takes ~5mins, after which the mouse is returned to its home cage. and will be performed multiple times (i.e. beginning, middle and end) per animal throughout the duration of the experiment. This helps us determine if our interventions have a beneficial impact on body weight loss.

Assessment of metabolic health: Mice will also be assessed for development of type 2 diabetes using glucose, insulin and pyruvate tolerance tests (GTTs, ITTs and PTTs respectively). GTTs will allow assessment of whole body glucose maintenance, ITTs will allow assessment of the animal sensitivity to insulin (eg. animals that are type 2 diabetic for example, will not respond to an insulin injection well as they become resistant to its effects) and PTTs allow us to specifically examine the effects on the liver's ability to handle glucose. They involve a fast (food withdrawal) normally for 5-hours (16 hours for PTT) and a bolus injection of either glucose/insulin/puryvate. Blood glucose is then measured at time points zero, 15, 30, 60, 90 and up to 120 minutes (when the glucose levels should be back to normal).

Assessment of pharmacological interventions We will assess whether pharmacological intervention improves health in obesity, disease or ageing by using drugs targeting proteins important in insulin/glucose action will change these responses. These drugs will either be injected under the skin (subcutaneously (s.c.)), incorporated into food/drink or injected into the abdomen (intraperitoneally (i.p.)). Treatment time will depend from a few hours (to test for acute changes) to weeks (normally around 16 weeks).

Assessment of the state of disease/Inflammation. In people living with obesity, diabetes or cardiovascular disease, there are increased circulating levels in the body of the bacterial pathogen lipopolysaccharide (LPS). To study what this may do in the body and in particular in a diabetic kidney/heart. Animals may be treated with drugs (eg. several hours before or days) prior to the LPS injection to test protective effects.

Cognitive Assessment. To investigate the effects of our nutritional or pharmacological interventions on animal cognitive or motor performance, which are known to decline in patients with neurodegenerative disorders such as different types of dementia (eg. most common one would be Alzheimer's disease), we would place them awake and freely moving animals into Y-maze tests, Phenotyper video recording cages or Rotarod apparatus. In a typical experiment, we would perform tests prior to any treatment (baseline) and then repeat these towards the end of the experiment in order to assess if our treatment has had beneficial effects. Animals would not normally be tested more than these occasions as they would not be "naive" to the treatment therefore careful analysis would have to be performed to know if the animal remembered the tasks versus treatment effects.

Pharmacological (STZ) induction of diabetes. To investigate the effects of diabetes on diabetes associated comorbidities, we propose to use a range of studies, from trying to understand how diabetes affects wound healing, to kidney health, eye health and metabolic health, as these are major complications of diabetes. To study these, we have to induce diabetes in the first place, the most common way to do so is using a chemical called Streptazotocin (STZ). After we induce diabetes, we can then study effects of diabetes on wound healing, kidney dysfunction, retinopathy. These experiments will allow us to determine if our interventions can protect against or revert changes induced by diabetes.

Imaging of animals. Animals may be subject to different types of imaging procedures to assess their health before and after our interentions. Some may not require anaesthesia, such as for example echoMRI, in which a free moving animal is inserted into a tube for a scan to determine percentage of fat mass versus muscle mass. This is particularly important for any treatments expected to a) improve obesity, b) improve metabolism (ratio of fat/lean mass may switch to more lean phenotype), c) STZ- diabetes treatment is known to lead to body weight loss (just like in patients with diabetes) but this will help us determine healthy vs unhealthy loss (eg. loss of too much lean mass would be unhealthy). To assess eye function in diabetes, we may use fundoscopy, which would allow us to visualise the eye and the retina of the eye.

Repeated Anaesthesia: repeated anaesthesia may be required in particular in experiments in Protocol 7, during diabetic wound healing experiments. Animals have to be anaesthetised for skin punch biopsy application (general anaesthesia) as well as every 2 days afterwards in order for animals to be asleep to taken wound tracing measurements (therefore measuring the speed/rate of wound closure).

Delivery of drugs using mini pumps: mini pumps may be implanted in Protocol 4 in cases where it would be more beneficial to mice to assess effects of hormones or drugs using a slow release system rather than injecting daily.

Single housing will be required during certain procedures such as measurement of energy expenditure, behaviour (phenotyper recordings) or in surgical experiments (to avoid wounding and fighting).

Food restriction may be applied in some cases up to 48 hours (Protocol 2) to assess hormonal influences during fasting/refeeding and normally around 5-16 hours (to assess basal and overnight hormonal changes). The prolonged (48 hr) fasting would only be done in an animal once if done at all. 5 hr fasting is normally required prior to glucose homeostasis assessment (such as glucose/insulin tolerance tests for example) whilst 16 hours normally for hormone determination (and normally in more diabetic animals such as ob/ob and db/db mice).

Long term administration of substances (by s.c.) may be required when testing the effects of antidiabetic/anti-obesity medication to assess longer term if these are beneficial (eg 16 weeks of treatment may give us an insight if beneficial effects may persist in patients for around 1 year).

Terminal Culls. All mice shall be culled via Schedule 1 methods or via regulated culls if for example tissues need to be collected in a specific manner (normally the brain has to be dissected very fast and preserved to assess changes).

What are the expected impacts and/or adverse effects for the animals during your project?

Breeding/Use of genetic altered mice

We do not expect any adverse effects during breeding of our genetically altered (GA) conditional and Cre lines. However, in some cases when breeding GA lines such as the BSCL2 (lipodystrophy model) or ApoE (atherosclerosis model), these may have higher rate of pre-weaning deaths. These will be monitored very closely and interventions such as breeding in isolator cages or backcrossing to breed from heterozygous would be introduced. These would also be reflected in the GA passports.

Metabolic and Cognitive Measurements

No adverse reactions are expected during experimentation. For tolerance tests, mice will be injected I.P. and blood collected from a single needle prick to the tail. This will result in mild discomfort that will be transient. Some drug interventions may result in weight loss during high fat diet intake, however this should be beneficial to the animal. For experiments in modelling atherosclerosis, either the ApoE-/- or LDLR-/- GA mice will be used. These will be obtained from commercial suppliers. For experiments in modelling different types of dementia, for example Tg4510, these will be obtained from commercial suppliers; some GA models of Alzheimer's disease are generated and maintained by the groups at our university.

Inflammation

The dose of LPS should only result in mild transient discomfort. We are only interested in the early development of inflammation which is not expected to have any impact on the welfare of the animals.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

50% of the animals are expected to be of mild and 50% of animals are expected to be of moderate threshold.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Obesity and ageing act at the whole-animal level and will depend upon coordinated interactions of multiple organ systems within live animals. Thus these fundamental biological processes cannot be studied realistically in any other manner, for example cell culture or computer simulations. However, we will carry out extensive literature searches throughout the time-scale of this project in an effort to continually improve and refine our studies, in order to avoid unnecessary repetition of studies, any undue suffering and ultimately to identify appropriate replacements for these animal studies.

Which non-animal alternatives did you consider for use in this project?

Wherever possible we will augment our findings in whole animals by using primary cell culture (eg. skin cells, liver and fat cells) or commercially available cell lines. Indeed, we have published many biochemical studies using liver and immune cell lines for example rather than live mice or primary cells, in order to test out different inhibitors or stimulators of inflammation and stress. In most of our experiments we conduct in the lab, around 70% are performed in vitro with the remaining 30% requiring in vivo confirmation to test whole body physiology effects.

Why were they not suitable?

In vitro systems do not recapitulate complex whole animal physiology, therefore animal experimentation is necessary.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Based on our animal number usage and home office returns for the past 5 years and considering increase in funding we obtained in last year alone, we estimated that we are going to require a slightly higher number of mice than in the current PPL. This has incorporated allowing early career researchers to work under my licence (under my approval and supervision) to allow them to develop their own independent laboratories and deliver on funding they received, with expectation that each individual will then obtain their own PPL.

I have extensive experience and published data in the area of metabolic health, cardiovascular disease, inflammation and cognition. These have been used as resources to calculate the numbers needed for experimentation. For in vivo studies a sample size of 8-10 mice is required per group for most physiological studies. This may be increased to 12 per group if some tissues are required for both histological and RNA/protein analysis. Both male and female mice are now expected by all funders to be used and will be used, as appropriate for the disease/experimental design.

Breeding will be required to develop and maintain GA animals from our conditional lines using tissue specific Cre transgenic mice. We will use the expertise from our breeding wing to ensure the appropriate number of breeding pairs are set up to allow lines to be generated with minimum wastage and genetic drift. To maintain appropriate numbers of mice for experimental cohorts, all breeding is directed by the PPL holder herself. Any surplus animals will either be culled, and tissues stored for future analysis, or offered to other users. We will also use our ‘non-crossed’ conditional mice as our wild type controls to ensure all mice are utilised.

Appropriate advice will be taken from suitably qualified statisticians within the institution in order that our studies are always undertaken using the minimal number of animals but retaining appropriate statistical rigour throughout.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

All animal studies are preceded by extensive in vitro experimentation to elucidate the molecular pharmacology of the pathways being investigated and the NC3R’s experimental design assistant consulted. We also have published extensively on the optimal number of animals required to perform each experiment. During terminal culls, most tissues are harvested along with blood, and stored until required, to ensure nothing is wasted and each animal used to its full potential. These tissues are also available to other collaborators within and out with our institute.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

At all points we will follow ARRIVE guidelines for all of our experimental set ups; blinding will be used whenever possible. However, for all the physiological testing, animals will be randomised in order to avoid bias. We will also consult the PREPARE guidelines to reduce waste and ensure reproducibility of experiments. Whenever possible, we aim to use both male and female mice to ensure relevance to the disease and explore potential sexual dimorphism in treatments.

Animal numbers bred for use on this Project will be minimised as far as possible by matching breeding to experimental requirements. Pilot studies, appropriate statistical

analysis and power calculations will be employed to refine the number of animals used. The methods chosen will generate the greatest amount of data for the fewest animals used. To maximise the information gained from a single animal we aim to perform multiple in vitro analyses on each animal using the tissues obtained from terminal culls. Any pilot studies will be run in such a way that they will be rolled into the main study wherever possible, so that they are not additional to the numbers ultimately required for the main experiment.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use C57BL/6 mice for all our experiments and all GA animals (ApoE-/-, LDLR-/-, Tg4510, tissue specific Cre, conditional knockouts) will be on a C57BL/6 background. This strain is used routinely for all experiments set out. For cardiovascular studies, ApoE-/- or LDLR-/- transgenics will be utilised since C57BL/6 mice do not develop atherosclerotic plaques. Furthermore, for LDLR-/- mice, require cholesterol in their diet to induce plaques, whereas ApoE-/- models spontaneously form these which can be accelerated via high fat/high cholesterol feeding. Tg4510 animals have an overexpression of human mutated Tau protein, representative of a mutation found in people with Alzheimer's disease, and develop AD symptoms with age.

For our metabolic measurements and experiments assessing atherosclerotic plaques formation, it will be required for mice to be fed a high fat/high cholesterol diet, to induce obesity and obesogenic pathologies. Type 2 diabetes development will be monitored via glucose/insulin/puryvate tolerance tests (GTTs, ITTs and PTTs) using the most refined ‘needle prick’ to the tail tip. In some studies where obesogenic diet is used, pharmacological intervention could result in weight loss. However, this would be a beneficial consequence of the study and provide proof of principle that our intervention could be exploited for future therapeutic development.

Why can’t you use animals that are less sentient?

Less sentient animals do not have the physiology to accurately recapitulate the human condition.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All procedures are the most refined and up to date methods to ensure minimal distress to the animal. Ear biopsies will be used to perform genotyping analysis as these give the least harm and stress to the mice. We will also test the possibility of using using for DNA extraction. For tolerance tests, we will use a single needle prick to the tail rather than snipping the end that was used previously. We have also opted to use the AlphaTRAK 2

glucometers during these tests as found this equipment to be more sensitive and use less blood than other models. Echo MRI scanning used for body mass measurements do not require anaesthesia (unlike DXA analyses) and is undertaken in <5mins per mouse, ensuring minimal discomfort.

For the induction of bacterial infection, mice will be injected I.P. with LPS/peptidoglycan which is less invasive and more reproducible than caecal ligation puncture.

Mice will be handled routinely, prior and during experimentation, using the cupping and tubing method and group housed unless separated for welfare reasons (i.e. fighting). In addition, our animals are normally bought in at the time of weaning and grouped at that time in order to minimise the risk of fighting.

In addition to refine the use of injections, our lab has been moving away, whenever possible, from the use of i.p. injections to the use of s.c. injections (eg for drug/treatment delivery).

For inducible knockdown studies, we incorporate tamoxifen in the diet to reduce potential side effects associated with injections.

We recently (2023) published the most refined model of STZ-induced diabetes that is least stressful to the animal and maintains stable diabetes status.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Guidance for the project will be taken from resources provided online by NC3Rs, ARRIVE, Norecopa, PREPARE (hubs and microsites) and LASA guidelines for administration of substances. This includes links to publications, other online resources, and video and training materials. The vast array of resources guide on the general principles underlying the experiments highlighted in this project, including anaesthesia, breeding strategy and numbers, and experimental design. Changes in current guidelines will be implemented to the project in line with current literature and published reports. We consult with experts to implement improvements in animal welfare. Local refinements will also be implemented. In addition we provide for the community better and refined methods with animal welfare as priority which we publish in open access literature.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We regularly receive updates in advancement from the NC3Rs newsletter. We will be kept up to date of any changes to procedures from our Named Information Officer and our Named Veterinary Surgeon. Additional training will be undertaken when required including reverification if needed. We will also consult with our experienced animal staff and Named Animal Welfare Officers to ensure the most refined practices are used at all times. In addition, our Named Information Officer is involved with several databases and forums to keep informed of any changes to procedure and there are joint online 3Rs events organised with other establishments.

102. Mechanistic studies of memory 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
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Memory, Synaptic plasticity, Synaptic signalling

Animal types	Life stages
Mice	embryo, adult, pregnant, neonate, juvenile, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to advance the mechanistic understanding of how memory is formed, stored, and retrieved in health and disease. Furthermore, this project will also study whether memory impairments can be restored in disease models.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Many diseases affect memory, including Alzheimer’s disease, mental retardation and post-traumatic stress disorder. Additionally, there is age-associated memory impairment that is considered ‘normal’ but reduces the quality of life in old age. Currently, there is no cure for

disease- and age-related memory deficits. This is because there is a lack of mechanistic understanding of memory. Our project will advance such mechanistic insights.

What outputs do you think you will see at the end of this project?

The project will advance insights into the molecular and cellular basis of learning and memory. These insights will advance the understanding how the brain acquires, stores, retrieves and maintains information. The project will also address how learning and memory abilities change with normal ageing and in mouse models of dementia and intellectual disability. Our project will also investigate mechanisms of memory erasure. Memory erasure is an important way of adaptation and if impaired it can lead to disorders such as post-traumatic stress disorder. Specifically, the project will lead to A) The identification of new memory mechanisms and establish whether these mechanisms are impaired in models of ageing, dementia and intellectual disability. B) The identification of proteins and pharmacological drugs to enhance learning abilities and to erase fear memory. C) Basic insight how memory is maintained.

Findings will be made available to other scientists through publications in peer-reviewed journals and presentations at scientific conferences and meetings.

Who or what will benefit from these outputs, and how?

When the project is completed other scientists in academia or in industry will have benefitted from these outputs. In the long-term we hope that some of the outputs can inspire clinical trials for treating memory impairment in diseases.

How will you look to maximise the outputs of this work?

As for our previous projects we will disseminate our new findings, which will include 'negative findings'. We will publish our research findings in open access journals and we will present our results at national and international conferences. Further, as in previous years we will continue to collaborate with experts in the field to maximise our outputs.

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.

Studies with mice are instrumental for advancing the mechanistic understanding of memory. This is because many mutant mice have been developed. For example, the international mouse knockout project provides conditional gene knockouts. These mutants are very valuable for studying the requirement of molecular processes underlying memory, since they allow for the generation of region- restricted, inducible manipulations. Furthermore, in the mouse targeted point mutations can be designed.

These mutations can address the function of protein modulation by, for example, phosphorylation, or they can properly model mutations causing memory dysfunction in humans.

Further, a large number of mouse models of disease are available, such as Alzheimer's disease transgenic mice.

Our studies will use juvenile, adult and aged mice for memory studies, because memory mechanisms differ at these ages.

Typically, what will be done to an animal used in your project?

Genetically-altered mice will be bred and maintained (Protocol 1). Some mice will be aged (Protocol 2 and 3). The majority of the studies in this project will use behavioural investigations with genetically altered and non-genetically altered mice (Protocol 3). The genetically altered mice will not have a visible impairment (such as impaired movement), as this would confound the specificity of our analysis of memory mechanisms. For some manipulations the mice will have to undergo surgery under general anaesthesia. In these cases, substances will be injected into discrete brain regions to specifically interfere with protein signalling that is involved in memory processes. During an approximately 1 hour-surgery we will typically make very small windows into the skull to get access to the brain. These small windows will be made only once, and they are made to insert a fine capillary/needle into the brain to inject substances, causing only minimal damage to the brain. In some cases a capillary will be cemented onto the skull to allow for flexible administration of substances. After recovery from surgery the mice will undergo behavioural testing. For behavioural memory testing the animals may have aversive behavioural tests such as footshock and Morris water maze. After behavioural testing the mice will be killed by a humane method and brain tissue will be taken after death for an in-depth analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

GA mice will bred and maintained (Protocol 1), sometimes until they are aged (Protocol 2 and 3). If moderate phenotypes, such as tumors, occur during ageing, the mice will be killed immediately.

Some of the mice generated under Protocols 1 will be aged in Protocol 3 for experimental testing. Aged mice obtained in Protocol 2 will be killed for post-mortem analyses. In Protocol 3 some mice will undergo local administration of substances into the brain under general anasethesia. Typically, such surgery does not last longer than one hour and the animal recovers within a day. After recovery the animal does not experience pain, discomfort, weight loss, fraility, or abnormal behaviour, other than having particular deficits in learning and memory. After recovery from surgery the experimental manipulation is expected to impair or enhance memory formation, memory storage and/or retrieval. The surgery per se will not affect learning and memory abilities. Most of the mice generated under Protocols 1 will also undergo regulated behavioural testing which involves aversive behavioural tests such as footshock and Morris water maze (Protocol 3). These aversive tests will cause transient discomfort but no physical harm. Mice may typically spend 30 min/day but no more than 60 min/day in exploratory or learning paradigms, for a maximum of 30 days.

Some mice will be killed by a non-Schedule 1 method for tissue collection to allow for research on post-mortem tissue (Protocols 1-3).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The severity of Protocol 1 (Breeding and maintenance of GA mice) is mild for all animals.

The severity of Protocol 2 (Maintenance of aged mice) is moderate for 10% of the aged mice.

The severity of Protocol 3 (Experimental manipulation and behavioural testing of mice) is moderate for 70% of the mice.

Overall, about 60% of all of the mice will experience mild severity and about 40% of all of the mice will experience moderate severity.

What will happen to animals at the end of this project?

- Killed
- Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Learning and memory are properties of the intact brain in a behaving animal so that the processes underlying learning and memory must be studied in living animals including humans. In comparison to studies with humans the animal experiments allow for sophisticated manipulations, which are essential to establish causality. For studies of learning and memory there are no suitable in vitro, in silico, or ex vivo tests which can replace the use of animals. Work with unprotected animals, such as fruit flies and nematodes, has yielded some insights into memory mechanisms. However, in mammals the underlying molecular and synaptic mechanisms are more complicated than in these unprotected species, justifying the use of mammals not only to develop a mechanistic understanding of memory but also to provide basic insights that can be used for developing treatments of memory impairment in humans.

Which non-animal alternatives did you consider for use in this project?

Searching the Pubmed database, I considered the following non-animal alternatives: 1) Human genetic data which have identified mutations associating with learning and memory dysfunction. In this project I aim to model some of these mutations in mice to develop an understanding how such mutations affect learning and memory. 2) Ultrastructural analysis of synapses in human post-mortem tissue from patients with memory dysfunction. In this project I aim to understand how such synaptic dysfunction can develop, using mouse models. 3) Molecular analyses in brain slices from animals or in cultured neurons from animals after electric stimulation and/or molecular manipulation. Such experiments are

informative about molecular processes which lead to synaptic dysfunction. In this project I aim to confirm that candidate molecular processes are relevant after behavioural training. 4) Computer modelling which predicts the impact of particular synaptic changes for memory. In this project we will generate more data for further more realistic computer modelling.

Prior in vitro experiments will establish that acute molecular manipulations are effective. For example, knock-down approaches will be optimised with cultured cells and only the optimal knock-down approach will be used with mice.

Why were they not suitable?

Human genetic data provide some insight as to which molecular processes are involved in learning and memory. However, there is only a limited set of known human mutations which associate with learning and memory dysfunction. Most human mutations are point mutations which do not inactivate gene function. But it is fundamentally important to study knockout mutations as these define the function of a gene of interest. This will be done in our project in mice. Further, the human genetic data do not reveal which synaptic plasticity is important for learning and memory. Therefore, it is essential model human mutations in mice, which will be done in our project, in order to study the synaptic cause of memory dysfunction in human diseases.

Ultrastructural studies with human post-mortem brain are informative, but because no experimental manipulation can be undertaken it remains unclear how important the observations are for learning and memory. in nature as experimental manipulations cannot be undertaken. In contrast the proposed mouse work will test whether ultrastructural features of synapses are important for memory. For example, using pharmacological manipulations it can be tested whether multi-input synapses (which can only be detected by ultrastructure) are essential for memory storage.

In vitro molecular analyses are informative, but they are prone to artifacts, as the tissue preparation induces injury responses which do not take place in the intact brain and because cultured neurons are not connected and supported in the same way as in intact tissue. Therefore, the proposed studies are needed to establish molecular mechanisms in the intact brain underlying learning and memory.

Computer modelling provides theoretical predictions about learning and memory processes by using current knowledge. However, computer modelling did not predict that multi-synapses are essential for memory formation in old age. This knowledge was obtained from our previous mouse work (Aziz et al., 2019). Our project will continue to provide more novel data, which afterwards computer modelling can implement to generate more realistic models of learning and memory.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 design and methods of analysis of the results have been taken from the Statistical Services Unit. Where relevant, factorial experimental designs will be used, rather than the one-thing-at-a-time approach, to maximise the information obtained from the minimum resource. For most 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 will use our previous experience (ours, or from the literature) to select sample sizes. In terms of the numbers of animals required, we expect that 10-15 animals per group should be sufficient to reach the scientific objective. Based on our previous HO returns I have estimated that a maximum of 2500 mice will be used in this project.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Prior in vitro experiments will establish that acute molecular manipulations are effective . For example, knock-down approaches will be optimised with cultured cells and only the optimal knock-down approach will be used with mice. If multiple treatments will be studied a common control group will be used in order to keep the animal number at a minimum.

Inbred or hybrid mouse strains will be used in order to reduce variability between animals.

As principles of good experimental work, we will follow the guidance on experimental design on the NC3Rs Experimental Design Assistant website, which includes power calculations to determine the sample sizes per group, randomization and blinding (masking) in order to exclude bias in the experiment, accounting for sex in the randomization, use of dedicated procedural rooms for the experiments, consideration of different types of intervention, and use of data transformation to obtain normalisation so that parametric statistics can be applied.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Our mouse breeding strategy will avoid unnecessary generation of mice. We will share tissue samples with colleagues.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 GA mice to address mechanisms underlying learning and memory. These mouse models have mild phenotypes so that behavioural assessment of learning and memory can be undertaken. Additionally, we will use the best mouse models for memory dysfunction in disease, such as dementia. For example, recently estalished APP knockin

mice model best familial Alzheimer's disease. At the time of behavioural testing these mice have a mild phenotype.

We will use behavioural testing which has been refined over the last 30 years. Importantly, we will use minimal training to address the mechanistic analysis of memory.

Why can’t you use animals that are less sentient?

We will use primarily young-adult and aged animals for behavioural testing, because our research question is about memory mechanism at these ages. It is known that adolescent animals use different mechanisms for learning and memory, in comparison to young-adult and aged mice.

Even though Drosophila and C. elegans are less-sentient species which have been used for learning and memory studies, they do not sufficiently address the mammalian complexity. For example,Drosophila does not have dendritic spines, which is a very important synaptic compartment for learning and memory processes. Further, the molecular complexity is too simplistic in Drosophila in comparison to mammals. For example, there is only one CaMKII gene in Drosophila, whilst there 4 CaMKII genes expressed in mouse and human brain (CaMKIIalpha, CaMKIIbeta, CaMKIIgamma and CaMKIIdelta). Importantly, these different CaMKII genes have distinct functions at synapses.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will follow the guidance of our NVS to refine surgical techniques and post-operative care and pain management. Aged animals will undergo increased monitoring in order to identify adverse effects early, such as tumours. Additionally, we will follow the literature to identify refined methods for behavioural testing.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the following, published best practice guidance:

For studies with aged mice

Progressing the care, husbandry and management of ageing mice used in scientific studies - Michael JA Wilkinson, Colin Selman, Lynn McLaughlin, Linda Horan, Lindsay Hamilton, Colin Gilbert, Caroline Chadwick, J Norman Flynn, 2020 (sagepub.com)

For drug administration https://doi.org/10.1258/0023677011911345

For aseptic surgery:
https://www.lasa.co.uk/PDF/LASA_Guiding_Principles_Aseptic_Surgery_2010.2.pdf

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will read about the latest research in our field and if novel, improved mouse model of disease become available, then we will use them as soon as possible.

A 3R officer who will inform us about advances in the 3Rs, so that we can implement them as soon as possible. Additionally, I will attend 3Rs relevant meetings and study the monthly NC3Rs newsletter.

103. Regenerative Medicine for the Upper Aerodigestive Tract

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Regenerative medicine, Gene therapy, Airway surgical research, Head & neck cancer models, Patient- derived xenografts

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?

This project aims to continue advancing our group's understanding of regenerative medicine strategies for use in the tissues of the head and neck. Experiments performed under this licence to implant combinations of cells and/or replacement tissues will help the transition of our work to reach first-in- man studies and clinical trials, as well as retrospectively examine key elements from the clinical setting requiring further optimisation.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

This work is vital to help us continue optimising translational treatments for the airway and the head and neck. Without work such as this, there will less forward motion in cutting-edge care for severely affected head & neck and airway patients of all ages who do not respond to conventional therapies. This work adds to the growing bridge of data and evidence between preclinical and clinical regenerative medicine therapies.

Work performed under our group's licences preceding the current application has greatly helped in our optimisation of tissue-engineered airway transplantation, to the point where the latest recipient is clinically normal at the 5-year follow-up mark. It has also helped us to examine multiple key elements of what is a complex multi-faceted strategy for personalised patient care, by answering questions relating to rare clinical scenarios that can't be looked at in any other way. Several other projects within the group are at the cusp of first-in-man translation, including gene therapy for airway cell diseases in childhood, and the UK Medicines and Healthcare products Regulatory Agency (MHRA) are clear that further animal studies are key to demonstrate a safe proof-of-concept prior to them considering granting us regulatory approval. This work is therefore completely essential to our ongoing research programme.

What outputs do you think you will see at the end of this project?

We envisage this project to generate key new information across a number of 'missing links' in our group's regenerative medicine strategy, which we think will allow us to move towards established use for patients towards the end of this licence's lifetime.

We had a planning meeting with the MHRA in December 2021 to discuss setting up a clinical trial in personalised airway cell grafts for voicebox and windpipe scarring, which is uncommon but extremely severe and difficult to treat – they reported that rabbit studies to optimise the cell engraftment process within the airway was the missing link experiment in our pre-clinical dossier of safety information. This work will be performed early in this licence's tenure. Likewise, key studies into cell medication injections within the voicebox are the missing link to our establishing a large clinical trial into this therapy for vocal cord wasting, a very common condition seen with in the older population.

Other new key information that we think is achievable within the next 5 years includes data about how the immune system affects and drives airway healing and scarring, and the effect that immune modulating medication might play on this process. This could realistically also lead to first-in-man clinical trials of topical therapy for airway granulations (a major problem within airway surgery) within 5 years, as existing medications and delivery devices are already commercially available could therefore have their use extended to cover the upper aerodigestive tract.

Further discovery science performed as part of this project also includes the optimisation of 3D-printed personalisable airway stents.

Who or what will benefit from these outputs, and how?

The primary benefit is hoped to be to patients. If successful, the strategies we will work on under this project could lead to a scene change within our field of airway surgery by reducing the number of patients with mature airway scars who require tracheostomy (and are at high risk of death from tracheostomy issues) and/or open surgery (with its repeated intensive treatments and hospital stays). Treatments that reduce the combined burden of

these conditions on the healthcare system have the potential to save the NHS millions, as well as greatly improving affected patients' quality of life and reliance on costly social care packages.

Following the success of our most recent successful windpipe tissue-engineered replacement case, who is living a normal symptom-free life 5 years post-surgery, we will continue to optimise all of the component parts of our regenerative medicine strategy so that windpipe and voicebox partial replacements remains a viable therapy for the worst-affected adults and children.

How will you look to maximise the outputs of this work?

We will continue to present our findings at relevant National and International conferences and to write up our approaches for open-access publication (regardless of success - negative results will also be presented to better inform the field). In keeping with the truly multidisciplinary nature of regenerative medicine, we will continue to nurture and build our networks both within our group and the wider specialist clinical units who care for these patients.

Species and numbers of animals expected to be used

- Mice: 360
- Rats: 275
- Rabbits: 120

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The purpose of this body of work is to test regenerative medicine constructs that are in late stages of development, prior to use in the clinical setting. Part of this testing process must be to test the constructs in mammals and in their intended body location within the head and neck.

Animals who are to receive constructs containing their own cells will necessarily have had to undergo prior harvest of these cells. Animals who are to receive human cells, or where the role of the immune system in healing is being examined, will need an altered immune system. This will either be using particular strains of mice and rats with lowered immune barriers or with the use of medication in rabbits where no such strain exists.

For preliminary safety trials of specific constructs, adult mice and rats will be used. These are likely to be stable under anaesthesia and are sufficient to answer the majority of scientific questions about how well the constructs perform in the body. The small size of the rodents also allows for non-invasive monitoring of cells within constructs over time. Where a tissue flap is likely to be required in the clinical context to support the construct, this will be modelled by placing constructs into muscle or abdominal tissue wraps in rabbits, whose larger size means they can accommodate graft constructs with lower overall tissue distortion and discomfort.

Where entering the airway may be done with a low risk of bleeding or swelling, the rat will be used. However, most surgery to the airway comes with increased risks on smaller airways and so where the intended construct location is the voicebox or windpipe, rabbits will usually be used. Young adult rabbits have airways of consistent and comparable dimensions and shape to human babies, making it straightforward and reliable to manufacture constructs for particular animals, as well as rendering the model extremely realistic when designing surgical techniques.

Despite the increased surgical ease of using male rabbits for neck surgery, we will primarily use female rabbits who are at lower risk of fighting and can therefore be group housed postoperatively with a better standard of welfare and lower risk of injury to their surgical sites.

Typically, what will be done to an animal used in your project?

Under Protocol 1, animals will typically undergo a 20- to 30-minute procedure under general anaesthesia, where they have the organ of interest exposed and a small amount of tissue removed to establish individualised cell cultures. In the airway, this would equate to roughly a 1-2mm2 laryngeal mucosal biopsy or 1-2 tracheal ring rings to harvest the airway lining and cartilage. Where possible, the anterior-only portions of tracheal rings are harvested so that continuity of the posterior wall is maintained and dissection is limited. Painkillers will be given to minimise suffering. Animals will be left to heal and recover whilst their cells are grown in the laboratory, labelled for tracking and spread onto a scaffold and/or delivery material. This process takes around 3-5 weeks, during which the animal is living without interventions.

In experiments where the placement of human cells need to be tested, mice or rats with naturally altered immune systems may instead receive human cells or cells from another individual. In rabbits, where no such line naturally exists, these experiments will involve prior establishment on individual animals on medication by daily administration of immunosuppressant medication. The choice of immunosuppressive agent will be modelled on the clinical scenario as far as possible – where an oral formulation of an agent exists, this will be preferentially used. In the case of agents where no oral formulation exists, subcutaneous injections. may be required. Occasional blood sampling may be required during this time to check adequate drug levels.

Under a second general anaesthesia episode with recovery, animals will undergo implantation of a construct, containing their own previously harvested cells, into either a generic body position with excellent blood supply or into the correct anatomical location within the head & neck/airway. In some animals, an injury to the area in question may have been made 7-10 days prior to this second procedure (which will take 15-20 mins to perform) and the construct used to repair the injured organ. This procedure usually takes around 45-60 minutes.

In experiments where grafts require ongoing additional support to gain a blood supply from surrounding healthy tissues, grafts may be implanted ahead of time into a flap of muscle or abdominal tissue and moved into the correct anatomic position at a third surgery around 2-3 weeks later together with a bridge of this tissue. This procedure takes around 45 minutes.

The constructs will be monitored within the animals using a combination of methods. Animals who have received constructs containing labelled cells will undergo periodic non-invasive imaging of these cells through their skin. Animals undergoing airway surgery will undergo monitoring and troubleshooting using keyhole camera equipment under short general anaesthesia episodes (<30 mins) on a weekly basis or sooner if airway difficulties develop.

At the end of the experimental period, animals will undergo a final period of general anaesthesia without recovery.

What are the expected impacts and/or adverse effects for the animals during your project?

We have found, in our extensive previous experience, animals recover well from these procedures with low discomfort and minimal airway concerns. Animals undergoing voicebox surgery may find swallowing painful to start with, but we have optimised our painkiller timings to minimise this and find that in the majority of surgeries, animals return to being active, eating and drinking on the same day, much like children undergoing similar procedures in hospital.

The main anticipated adverse effects are those around the time of surgical procedures themselves, those due to generic wound issues or those due to the wound's specific location – these may be early or occur gradually as the experiment progresses over a number of weeks. At the time of surgery, given the goal in airway anaesthesia is to walk the line between keeping the animal asleep enough to operate but still light enough for the animal to breathe for themselves, the anaesthesia may be transiently too deep or too light over a period of seconds to minutes but is under continuous monitoring to make sure that anaesthetic delivery is matched to the animal as far as possible.

In the first few days following any open surgery, animals may suffer pain and discomfort from surgical wounds – this is alleviated as far as possible with painkillers. Animals may also develop wound infections, which are managed with antibiotics. Those animals who are lacking in an immune response are at higher risk of developing infections and will be pre-emptively given antibiotics to reduce this risk.

At each planned camera check under general anaesthetic, rabbits may suffer pain on anaesthetic induction due to subcutaneous or intramuscular injections of anaesthetic medication, following which anaesthesia is maintained through much less painful IV routes. Endoscopy and/or imaging procedures should not in themselves generate significant pain or adverse effects.

Animals receiving airway constructs may develop trouble breathing from early bleeding or later problems with narrowing due to scar development – these should improve following endoscopic treatments, which are much less painful than open surgery. Trouble breathing that does not respond to treatment will prompt termination of the animal to prevent 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)?

All animals used in this project will suffer moderate levels of harm.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Our work is highly translational, focussing on bridging the gap between the bench and first-in-man patient benefit. We have approached the Medicines and Healthcare products Regulatory Agency (MHRA) with our existing data and, whilst they have been very encouraging of our approach, they have made it clear that they consider further animal studies as key to allowing us to progress our goals towards the bedside.

Which non-animal alternatives did you consider for use in this project?

Many non-animal alternatives are, at least in part, suitable to answer parts of our scientific questions, and we remain committed to replacing animals with alternate means of gathering required information wherever we can. Our wider group has extensive experience with bioreactors and culture dish-based systems that aim to simulate the body's environment, and these systems will be fully employed prior to experiments in animals. They will also inform the conditions of animal experiments, as well as reducing required group sizes to include only those that have shown sufficient prior promise.

We are also committed to gleaning retrospective information from clinical cases whenever possible, and patients’ families are regularly asked without pressure, and agree to, to involve their children in research where it does not impact on their clinical status.

Why were they not suitable?

Ultimately however, no computer- or lab-based modelling system currently exists that can accurately predict the interactions between cells, scaffolds, grafts and host with sufficient complexity to evaluate the healing and function of implants within complex organs. It is hoped that experiments such as these will help inform computer-based modelling systems of the future, and all information gained that might be useful in that capacity will be uploaded to open access data repositories.

Whilst retrospective evaluation of clinical cases is vital to fully learn from each outcome, there are many individual factors for each individual case that prevent robust and generalisable conclusions from being drawn.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design

studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Mice

Given the diversity of parameters that need optimising within each Advanced Therapeutic Medicinal Product (ATMP), I estimate that at in any given month an average of 1-2 batches of 3 mice will undergo ATMP implantation, rounded up to cover for any issues arising from experiments with immune-altered animals. In those cases where animals are to receive their own cells as part of the ATMP, they will also undergo cell harvest, but there are very few scenarios in which an animal will solely undergo cell harvest.

Rats

Given the diversity of parameters that need optimising within the ATMPs, I estimate that at in any given month an average of 1 batch of 3 rats will undergo ATMP implantation, with an additional 10% animals estimated to cover for any loss from immune alteration problems. Additionally, we have funding for 6 batches of 3 rats to undergo voicebox surgery, and if these prove successful, further funding will be sought to continue these experiments. In those cases where animals are to receive their own cells as part of the ATMP, they will also undergo cell harvest, but there are very few scenarios in which an animal will solely undergo cell harvest.

Rabbits

The majority of animals will require prior procedures to collect cells to make the ATMP, and/or to modify their immune system to accept the graft/influence wound healing, and/or to pre-implant the ATMP. However, all animals will undergo ATMP implantation in the upper aerodigestive tract. We have funding to run 5 batches of 3 rabbits undergoing windpipe procedures within the first year of the licence, as well as to commence voicebox surgery in a similar number. Should these prove successful, further funding is highly likely to enable experiments to continue at a similar rate.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have generally run our ATMP implantation experiments in pilot batches of 3 animals - this is both to allow for close monitoring in the post-op period and because our non-invasive imaging of cells within grafts allows us to answer many questions regarding tissue regeneration within the same animal over time.

We then amplify group numbers with further batches only where this is required for statistical analysis once an ATMP strategy has been initially shown to be highly promising in terms of safety and function.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Each animal will be analysed in as many ways as possible without increasing animal suffering to reduce the numbers needed for experiments (e.g. monitoring, non-invasive imaging, airway camera checks, analysis of organs). To reduce bias, animals will be ordered in batches from the same suppliers according to a specified weight and sex.

Prior to airway surgery (rabbits only), grafts will be randomised to experimental/control groups and prepared for surgery by a separate researcher, such that surgeons do not know the seeded status of a given graft during surgery, follow-up and post-mortem analysis. The ability to take endoscopic biopsies of grafted areas to look at cell engraftment and integration over time enables the same animal to receive follow-up at multiple time points without the need for early termination points, as well as controlling for variability in individual animal/graft interactions. Experimental tissue will be paired in analysis with control areas of the animal's own airway. Video footage will be taken during endoscopic procedures to enable repeat assessment by a second blinded surgeon.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Candidate ATMP designs are tested as far as is possible in the ’dry’ setting prior to animal testing to ensure that only those most likely to succeed are used. However, this process is not infallible, as blood vessel growth, immune response and cell survival (the most difficult parameters to predict in the lab) may prove poor in certain combinations. Therefore, for initial animal experiments to confirm cell culture findings, mice and rats will be used with grafts in a general non-airway position. Rats are more appropriate than mice where more than two graft types are to be tested against a control, as their larger size allows for both experiment and control grafts to be accommodated within the same animal without significant animal discomfort.

Inbred strains of mice and rats are the appropriate species for this as they are economical to keep and are the lowest order species evolutionarily to provide useful data on interactions between implants and the environment of recipients’ tissues. Grafts will be placed in subcutaneous positions (the most comfortable and easily accessible location), unless the scientific rationale for using the more painful intraabdominal or intramuscular positions in a particular experiment is compelling (i.e. better blood supply is necessary, or the graft’s final position is intended to be in muscle of the human head & neck, or it is usual clinical practice to support repairs in that area with a flap of tissue with a good blood supply). Following analysis of these data, there will likely be further 'weeding out' of less-than-perfect strategies before they are tested in an airway position.

For surgical work specific to replacement/repair of the airway, which serves as the gold standard for whether grafts function in their intended role, we will use rabbits according to our well-established model of airway surgery and reconstruction. Rabbits are an

appropriate species for a airway surgical model for children as they have airways of comparable size and morphology to human babies.

Specifically, New Zealand White rabbits demonstrate low individual variation in windpipe diameter, minimising the chance of graft/recipient size mismatch. Longer surgical follow-up is achievable than in rat or mouse tracheal surgery, as commonly available ‘small animal’ veterinary keyhole camera equipment can be used to alleviate distress and reduce complications.

In cases of minimally invasive voicebox surgery, such as injection therapies, we will use a rat airway surgical model commonly in use with very low mortality at other international units performing airway research. The surgeons using these models will demonstrate procedural competency prior to commencing their work to reduce any animal harm from the surgical learning curve.

Why can’t you use animals that are less sentient?

Under previous project licences, we have striven to refine our use of animals to the minimum required to answer each scientific question most effectively and will continue to do so under this licence. We use rodents for early low-risk/low-discomfort experiments, only progressing to rabbits when suitable strategies with a good chance of success have been identified and optimised.

We feel strongly that it is not practical to perform extensive open surgical replacement in rodents, as even with perfect surgical technique, the risk of sudden death from airway blockage is unacceptably high in the post-operative period (from lining swelling, blood clots or mucus plugs). Several groups report using rat models of open airway surgery, particularly in the US, but they are willing to accept levels of animal loss that we would find unacceptable, with up to 50% requiring early euthanasia in some studies.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have worked within our institution since 2013 to establish our rabbit model of airway repair/replacement and optimise the amount of pain, suffering, distress, or lasting harm each animal experiences. In particular, we have carefully optimised our anaesthetic and pain management protocols, as well as our surgical techniques and the follow-up evaluation/treatment of pending issues. This refinement work is in partnership with local and regional NACWOs and NVSs and other international researchers expert in the model. Under my other project licence, we have continued to optimise anaesthesia protocols as well as improve our endoscopic treatment capabilities and institutional expertise. We have also made improvements in the husbandry and welfare of the animals pre- and post-operatively, such as in using female rabbits to allow group housing and social interaction/play rather than the surgically-easier male, and will continue to work to do so over the lifetime of this licence.

At all stages, we have found a good tolerance of the surgery itself, with all surgical controls performed under either research HO licence surviving symptom-free until their planned timepoints. This demonstrates that the surgery itself is well-tolerated and that the main factors affecting experimental outcomes are those emanating from the grafts to be tested.

Unlike the comparable scenario in clinical practice, intensive care facilities are not practical to provide in a laboratory animal setting. Therefore, the risk of sudden severe respiratory distress from mucus plugging in animals receiving airway ATMPs (who, by definition, have a length of airway with compromised mucus clearance), or the potential risk of them being found dead in the morning despite appearing completely well at their evening checks, cannot be completely removed. The severity limit for our rabbit airway work under a previous licence was ‘severe’ in case this occurred again, however due to ongoing refinements and increasing expertise we encountered no further incidences of animals suffering sudden respiratory distress and therefore and we feel that returning to a severity limit of ‘moderate’ on the corresponding Step of this licence is therefore justified.

Any animal who is noted to have respiratory distress is urgently evaluated that working day by the researcher, and if warranted, undergoes endoscopy under anaesthesia to diagnose and treat the cause. Animals are terminated immediately following discussion with the researcher where the researcher is not available to attend in person within this time frame. We have seen throughout our previous work under previous project licences that, in cases where symptoms are more gradual in onset, that moderate respiratory effort will usually have either resolved, stabilised or worsened to the point of requiring early termination by 48 hours. Humane end points will therefore be at a default of 36 hours following the first discovery of airway noise in animals who continue to decline in any way (louder noises, reduction in activity), although endoscopic assessment of these animals will help to further tailor endpoint timings to individual animals (i.e. less than 36 hours).

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the NC3Rs guidance on best practice with regards to laboratory animal anaesthesia, analgesia, intraoperative care and post-anaesthesia support, and ensure that researchers have undertaken the e-learning modules on the NC3Rs website that are relevant to their practice in this regard. We will work closely with our NACWO and NVSs to ensure that best practice guidance from them is followed in terms of animal welfare and environmental enrichment, and meet regularly to discuss any updates to practice. There is no best practice guideline for management of the surgical airway in rodents or rabbits and we intend to build on our previous experience as well as those available in veterinary practice covering airway management for other procedures to write a guideline for peer review.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will continue to cultivate our excellent relationships with our NVS, NACWO and the staff at the Biological Services unit, so that any advances that are communicated to them from a laboratory animal practice side are implemented. We already subscribe to the NC3Rs newsletter and regularly check in with the NC3Rs website to identify relevant training opportunities for personal and project licence holders.

Once advances are identified and evaluated as being relevant for our Protocols, I will ensure that all group leaders working under the licence have been informed and ensure they evaluate them for integration into their own practice, and continue to promote a group culture where improvements are taken up as default unless scientific rationales to keep the status quo in specific cases are compelling.

104. Blood vessel glycocalyx in health and disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Blood vessel, Barrier function, Glycocalyx, Kidney, Therapy

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged
Rats	juvenile, adult, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To gain greater understanding of the protective layer in blood vessels (glycocalyx) in health and disease so that therapies can be developed

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 glycocalyx is a gel-like layer on the inside of blood vessels that forms a barrier, keeping proteins and red blood cells in the blood. This layer is also important to allow the blood vessels to respond to changes in hormones and blood flow, such as in exercise, and to protect blood vessels from mild insults such as a high fat meal. In cardiovascular disease, this layer is damaged, which exposes the blood vessels to stress signals in the blood. Damage to the glycocalyx can play a key role in contributing to important diseases,

such as diabetes, kidney disease, atherosclerosis, and sepsis (blood poisoning). The purpose of this project is to increase our understanding of how the glycocalyx is built up and broken down normally and in disease. This will help us to develop treatments to recover it in disease states, reducing the severity of lots of diseases.

What outputs do you think you will see at the end of this project?

It is to be expected that the work will generate information that will increase our understanding of how the glycocalyx is built up and broken down. Through this new knowledge we hope to identify and evaluate new treatments that could be used to restore or improve the health of blood vessels during disease. The data generated and conclusions drawn from the work will be presented at scientific conferences and published in scientific journals.

Who or what will benefit from these outputs, and how?

In the short term the people who will benefit from this work are scientists who specialize in cardiovascular disease. In the medium term, the research findings will contribute to the identification of new treatments that could be used to restore or improve the function of the glycocalyx during disease and thus in the long term, benefit patients and the health care workers treating them.

How will you look to maximise the outputs of this work?

The findings of the work will be presented at national and international conferences and submitted for publication in international peer reviewed journals. All publications will adhere to the ARRIVE guidelines and give detailed descriptions of all animal experiments.

Species and numbers of animals expected to be used

- Mice: 6000
- Rats: 500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The outlined work will be undertaken using rats and mice . These species have been selected as previous studies have established that the mechanisms involved in the formation and regulation of the glycocalyx share many common pathways with humans. In addition, these species are the least sentient of those for which relevant vascular disease models have been established. The majority of studies will be conducted in mice which, in addition to being the least sentient species, have the added advantage that we can change expression of their genes using established methodologies.

Rats will be used for studies that cannot be undertaken for technical or scientific reasons using mice. Rats will therefore be used for the models where larger blood vessels are needed for the type of analysis required and for some disease models which are rat-specific.

Typically, what will be done to an animal used in your project?

The majority of animals will arrive from an external supplier. They will acclimatise for 7 days and will become habituated to handling by researchers.

A typical experiment will include approximately 7 steps. Non invasive measurements (e.g. urine collection) will be taken, which are not thought to cause any more than transient distress. Induction of disease will be induced typically by administration via the drinking water or by injection, causing no more than transient distress. No acute adverse effects to the disease-causing agents is expected.

Genetic expression may be induced bygiving agents by injection or within the drinking water. Glycocalyx modifying agents will be given by injection, oral dosing or osmotic mini-pumps (to replace daily injections, where appropriate), all of which are well tolerated. Disease (diabetes, kidney disease) is typically up to 12 wk and animals are expected to experience mild phenotypes. Non-invasive measurements are carried out weekly, that sometimes require recovery anaesthesia, including imaging (e.g. echocardiography and angiography). These cause no more than transient and mild distress. At endpoint, animals are terminally anaesthetised and perfused, organs removed and schedule 1 culled.

A small number of animals (less than 1%) will be used in sepsis models that have the potential to induce severe disease however, all animals undergoing these procedures will be closely monitored and killed if signs of more than mild suffering occur and the duration of the study limited to a timepoint below which animals are unlikely to experience more than mild suffering.

What are the expected impacts and/or adverse effects for the animals during your project?

The vast majority of mice used in these studies are expected to continue to appear and behave normally throughout the study period. Mice used for type 2 diabetes studies are expected to become obese and to urinate more than normal but to otherwise remain healthy. Animals undergoing surgery are expected to experience some pain upon recovery, which will be controlled using pain killers.
Following surgery, animals are expected to recover uneventfully and to resume normal behaviour within a few hours. The small proportion of animals used in sepsis models may develop mild signs of suffering, such as reduced levels of activity and piloerection but are expected to remain responsive throughout the study period. Any animal developing overt signs indicative of more than mild transient pain 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)?

Mice Mild 57%
Moderate 43% Rats
Mild 70%
Moderate 30%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The glycocalyx is a thin layer of material that coats the inner surface of blood vessels. It is responsive to the internal environment of blood vessels and alters with changes in blood flow, blood pressure, growth factors and reactive oxygen species. Whilst human cell culture models of the glycocalyx exist, and indeed will be used in the study to show mechanism or prove relevance to humans, the potential to replicate the physiological conditions that occur in the living animal using such models is limited.

Therefore, any findings made using such models will need to be confirmed in the living animals.

Furthermore, it is not possible to use cell models to investigate the response of the glycocalyx to disease, which is a major component of the outlined studies.

Which non-animal alternatives did you consider for use in this project?

Cultured human cell lines studies and human population studies.

Why were they not suitable?

Cultured human cell lines will be used as part of the work to study the underlying mechanisms regulating the glycocalyx. These cells express a glycocalyx, but the conditions under which they are cultured lack the flow, crosstalk and blood components, which influence the integrity of the glycocalyx. In addition, it is not possible to study the effect of disease on the formation and maintenance of the glycocalyx using cell culture models. Consequently, there is no viable alternative to the use of animals for the outlined work.

Human population studies on existing published data sets will be undertaken to confirm the relevance of the study to human health and disease, however it is neither feasible or ethical to undertake the manipulation needed to determine the underlying mechanisms regulating the glycocalyx in human volunteers.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Animal usage has been calculated (using power calculations) based on our past experience and publications. Also taking into account current funded research needs, in the different disease areas, and a prediction of future funding within the duration of the licence. Numbers include breeding colonies for mice, hence the increased estimated numbers.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have always based our animal numbers on power calculations, we also refine our outputs as we go. For example, we can use an assay that requires animal tissue, to study kidney function, instead of testing the animal itself. This leads to less rodents being used and more reliable data.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

For new experimental designs we will complete pilot studies, where necessary, to help optimise animals numbers required.

Animal usage will be minimised by undertaking

Paired assessment of whole organ and individual vessel function

Repetition of vascular measurements before and after a glycocalyx modifying therapy

Simultaneous measurements of glycocalyx depth and blood vessel function

Study of multiple vessels in the same animal

The use of control data from similar 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 disease models used in the outlined studies have been selected to reproduce specific aspects of human disease and have been characterised and refined under my previous licence, such that in the vast majority of cases the animals continue to look and behave normally throughout the study period. In order to induce some models, animals may undergo a surgical procedure performed under general anaesthesia. These animals are expected to recover uneventfully and to resume normal behaviour within a few hours.

Upon recovery, all animals will be given pain killers, which will be maintained until all signs indicative of pain have ceased. A small proportion of animals may be used in sepsis models. The duration of these studies is limited to ensure suffering is minimised (4 hours for the caecal puncture model and 18 hours for the LPS model). Animals used in these studies may show reduced levels of activity and piloerection but are expected to remain responsive throughout the study period. Any animal developing overt signs indicative of more than mild transient pain will be killed.

Why can't you use animals that are less sentient?

We need a functioning, developed cardiovascular system to mimic vascular glycocalyx changes that can be reliably related to human disease. In addition, a developed (not immature) animal is required for better susceptibility to disease progression.

Much of our work is carried out on anaesthetised animals that are not recovered. Where it is not, a fully functioning animal is needed to be able to measure organ function (such as echocardiography or retinal imaging).

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The procedures to be conducted have been extensively refined to minimise their impact on the wellbeing of the animals. Where relevant, animals are habituated to the handling and procedural techniques to minimise any associated stress e.g. prior training for blood pressure monitoring.

Hydrophobic sand is used to facilitate the collection of urine samples, thereby avoiding the need to use metabolic cages unless it is necessary, due to bladder shy animals or a large number of animals used at one time. The disease models used have been refined such that animals are not expected to develop overt signs of suffering and all animals are carefully monitored to ensure that any adverse effects are detected at an early stage and appropriate action taken to prevent suffering.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Generally, we follow PREPARE guidelines. The formation of the study is based upon a literature search and our own preliminary data from in vitro work and pilot in vivo work. We have excellent communication with the Animal Sciences Unit staff and we always complete a pre-study briefing to address legal and ethical concerns. We are committed to developing refined procedures for substance administration, sampling, sedation and anaesthesia, surgery and other techniques.

All surgery is conducted according to LASA guidlines.

For our rodent diabetic models we follow DiaComp guidelines:
https://www.diacomp.org/shared/protocols.aspx?model=4

For our sepsis models we follow a welfare/clinical sign scoring system to refine endpoints.

Body temperature and mouse scoring systems as surrogate markers of death in cecal ligation and puncture sepsis. Safiah H C Mai, Neha Sharma, Andrew C Kwong, Dhruva J

Dwivedi, Momina Khan, Peter M Grin, Alison E Fox-Robichaud, Patricia C Liaw. Intensive Care Med Exp. 2018 Jul 27;6(1):20.

We also use the following reference for best practise;

REFINEMENT OF ANIMAL MODELS OF SEPSIS AND SEPTIC SHOCK Lilley et al, Shock, 2015

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We are constantly refining our protocols; learning from personal experience, feedback/discussions with our animal services unit and from collaborations/discussions/presentations from world experts at conferences and from evidence -based literature. The personal licence holders undertaking the work all attend relevant NC3R events/workshops organised locally and regionally.