Home Office

Animals (Scientific Procedures) Act 1986

Non-technical summaries for project licences granted January - March 2024

Contents

1. Advancing understanding of Paget’s disease of Bone

Project duration

5 years 0 months

Project purpose

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

Key words

Bone metabolism, Bone cells, Genetics, Calcium metabolism, Microbiome

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

To gain greater understanding of the mechanisms by which genetic factors interact with environmental influences to cause Paget's disease of the bone.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 studies in patients with Paget's disease of bone (PDB) have shown that genetic factors play a key role in causing the disease and influencing its severity. Epidemiological studies suggest that environmental factors also play a role but it is unclear what these are. This project will define effects of candidate genes for PDB on bone renewal and repair and will evaluate the potential role of dietary calcium intake and the microbiome on the disease. The knowledge gained during these studies will provide greater understanding of why PDB occurs as well as casting light in the fundamental mechanisms which regulate

bone repair, potentially leading to new treatments for the PDB and other diseases where the renewal and repair processes in bone are abnormal.

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

The project is expected to yield greater understanding of the causes of Paget's disease, including the identification and function of genes that predispose to the disease as well as the potential role of environmental triggers for the disease, most notably, dietary calcium and vitamin D intake and probiotics. Outputs will include publications in medical and scientific journals, presentations at scientific meetings and at information meetings for patients with the disease. The programme of research may lead to the identification of products which can influence progress of the disease.

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

In the short term (2-5 years) the outputs from this project will benefit other researchers and clinicians working in the area of bone metabolism and bone disease. In the longer term (5-10 years) the knowledge gained could lead to the development of new treatments or dietary interventions to prevent progression of Paget's disease.

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

We will seek to publish and present the results of all outputs from experiments described in the project whether or not the experiments are successful. New knowledge will be disseminated through presentations and publications and in collaboration with patient-support charitable organisations such as the Paget's Association.

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.

The project will use mice as a disease model since previous research has shown that genetically modified mice can reproduce many of the features of Paget's disease observed in patients. They have also been employed to study the effects of drugs that are used for the treatment of Paget's disease.

The studies will primarily involve adult and aged mice as Paget's disease predominantly affects people more than 50 years and becomes increasingly more common with age.

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

Typically, animals will experience mild, transient pain and no lasting harm from administration of substances by injection using standard routes (intravenous, subcutaneous, intraperitoneal).

Animals will undergo changes in diet and be administered probiotics or vehicle by adding these substances to food or water. These procedures are not expected to cause distress or adverse effects.

Exceptionally, where it is not possible to administered substances in food or water, the substance may be introduced directly onto the stomach in a tube (gavage). This may result in mild and transient discomfort.

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.

Small samples of tissue may be removed from the ear or tail on a single occasion to evaluate the identity of genetic mutations in mice used for the studies.

Animals may also undergo imaging by procedure called microCT scanning (a type of x-ray). If this is required it will be done under anasthesia and repeated no more than twice during the animal's lifetime.

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

Animals undergoing blood sampling, tissue sampling of the ear or tail and those being administered substances by injection may experience mild, transient pain and no lasting harm. Similarly animals that require to have substances administered by gavage my experience transient discomfort.

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

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

Mice: 80% mild, 20% sub-threshold.

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

- Killed
- Used in other projects

Replacement

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

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

Animal experiments are essential to investigate the the effects of candidate genes and dietary manipulations in the whole skeleton. Although it is possible to gain many insights into the effects of genes on the behaviour of bone cells from studies in tissue culture, methods have not yet been developed that can accurately reproduce the complex

interactions between diet, genetic influences and mechanical loading that are operative in the whole animal *in vivo*.

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

I have considered the use of computer modelling, cell and organ culture. Cell and organ cultures can address some of the scientific questions to be addressed in this project but cannot fully substitute for the use of animals. The use of species such as nematodes or fruit flies have also been considered but since these do not have a skeleton they were considered unsuitable.

Why were they not suitable?

This project application forms part of a larger programme of research that involves clinical trials in humans, molecular genetic studies and cell culture studies. The experiments detailed here are those that cannot be addressed by these alternatives.

Reduction

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

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

The estimated numbers for individual experiments looking at skeletal phenotypes in genetically altered mice uses the typical variations from previous experimentation to calculate minimum numbers of animals to be used whilst ensuring that the results are statistically significant. These calculations typically show that we need group sizes of 12-14 to achieve the quality of results we need.

The estimated number of animals that we will need to use for breeding is derived from our previous annual return of procedures data on a similar project

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

We employed the NC3Rs' experimental design guidance and experimental design assistant (EDA) to plan our experimental design, practical steps and statistical analysis utilising the advice and support for randomisation and blinding, sample size calculations and appropriate statistical analysis methods. We will use the EDA diagram and report outputs to support experimental planning with animal users.

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

At the end of the experiment, we will harvest as many tissues as possible at post-mortem. If we don’t need to analyse the tissues immediately, we will freeze them and make them available to other researchers working on similar questions.

Refinement

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

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

Mice will be allowed to grow old to study the skeletal responses in the ageing animal as this is most relevant to the human disease. To induce changes in the potential environmental triggers implicated in the human disease, some animals will be given substances by mouth, injection, or through food.

Injection may be necessary to try and replicate delivery of some of the interventions that are used in clinical practice. Rarely, gavage may be employed to study the effects of interventions that are given by mouth in humans.

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

Non-mammalian animals are limited in their use either because they do not have a skeleton or because their biomechanical environment is too different from the human immune system to provide reliable and clinically relevant results. We can’t use embryos or very young animals as Paget's disease is predominantly affects older adults.

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

Ageing animals will be carefully monitored by staff trained to work with ageing animals. Group sizes in ageing experiments will be increased to accommodate for loss of animals and to avoid single housing due to animal losses as the result of old age. Longer drinking spouts will be used, and animals will be monitored for adverse effects such as changes in weight, dermatitis, piloerection, paleness, changes in mobility, lumps, eye defects, abnormal respiration, or stools. If these are observed animals will be treated accordingly, and animals that develop severe effects will be humanely killed.

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

We will follow the principles described in the PREPARE guidelines to assist with planning animal research and testing and use the ARRIVE guidelines for reporting the results of experiments.

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

Example: We will regularly check information on NC3Rs website, we’ve signed up to the NC3Rs newsletter, we will meet the NC3Rs Regional Programme Manager, and attend Regional 3Rs symposia.

2. Behavioural and physiological effects of fish social 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 biology, host-parasite interactions, climate change, ecophysiology, behavioural ecology

Animal types	Life stages
Blue-green chromis (Chromis viridis)	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 will investigate how environmental conditions and parasite infection alter the physiology and behaviour of fishes, and whether the type of habitat (tropical, temperate) alters fishes' strategies to enhance resilience.

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

Why is it important to undertake this work?

The world is undergoing one of the most rapid changes in environmental conditions in geological history, with rising temperature and atmospheric CO2. In the marine and freshwater environments, concurrent changes in oxygen availability, pH, and concentrations of pollutants are presenting unprecedented challenges to the resilience of fishes. These environmental changes lead to altered prevalence of parasites. While parasites are a natural part of any healthy ecosystem, altered environmental conditions

are leading to changes in the landscape of parasite risk for fish hosts. Now more than ever we need to better understand how these changes are impacting wild animal populations, particularly their ability to thrive and survive in their environment, including their ability to find food, escape predators, and attract mates. Studies in this area will improve our ability to mitigate environmental changes on fish populations by enhancing the effectiveness of conservation policies.

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

This project will increase the knowledge on how the environment and parasites alter fish behaviour and physiology in the wild, which will be disseminated to the scientific community through peer-reviewed publications and presentations at scientific conferences. These results will also be communicated to a wider public audience through publication of popular science articles and engagement with the media (e.g., podcasts, print media).

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

My work on how parasites alter individual physiology and behaviour comes at a time when policy- makers and the public have a better appreciation than ever about how infection can influence hosts, given the recent covid-19 pandemic. Further, the world is entering a pivotal time to address the climate crisis, and my research on fish resilience in a changing world is essential to help us ensure persistence of economically and ecologically important fish species. Thus in the short term, this work will benefit the scientific community for understanding how global change and parasites alter fish behaviour and physiology. In the medium and long term, these results have the potential to inform policies for dealing with novel parasites and pathogens and mitigating the impacts of global change.

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

I have a wide domestic and international network of research collaborators who I will work with to ensure the widest applicability of these findings. I also plan to publish these findings in peer reviewed journals, even if the results prove non-statistically significant.

Species and numbers of animals expected to be used

- : 2000
- : 1000
- : 1000
- : 1000
- : 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.

Fish present ideal models to test questions related to sociality and they display highly diverse social behaviour within and across species, so results ascertained in fish species can be generalized to a range of other social taxa. Further, fish species are both an ecologically and economically valuable natural resource that suffer under biotic and abiotic

stressors, like those that will be studied here. Thus, this project will also provide evidence that will aid in the protection of fishes' function in important components of natural food webs in marine system as well as important food resources for humans.

We will mostly use the juvenile stage of fish as they are easier to maintain in a laboratory setting for many species and are yet to reach sexual maturity, at which point behaviours begin to change due to sexual selection and competition.

We have included in this project license one coral reef fish species (Chromis viridis) and a number of species of mullet (Chelon labrosus, Chelon ramada, Mugil cephalus, Liza aurata). The coral reef fish species (C. viridis) was chosen as it is a highly abundance and gregarious fish species found through the Indo-Pacific region, known as the 'Coral Triangle', where the diversity on coral reefs in orders of magnitude higher than coral reefs elsewhere in the world. This species has both a huge of amount of background literature that we will build on and is accessible for ordering from Oata accredited suppliers for the aquarium trade.

We will compare our findings in this coral reef species to a temperate mullet species. Species are highly social, found living in large social group in nature. Mullet are one of the most common inshore fishes in the UK. The species of mullet that we will use will be subject to availability during our studies. As all mullet species listed exhibit a high degree of similarity in behaviour, we can use any of these species for our studies.

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

For these studies, fish will either be order from an OATA-accredited supplier (Chromis viridis) or they will be collected locally along the south coast of England (all mullet species). Mullet will be collected using two pole seine (3 mm mesh and 3x2 m dimensions), cast net (5 mm mesh and 3 m diameter) or trap (pinfish trap: volume up to 8 cubic feet) from the shore. All methods have proved effective for capturing the study species listed here. All non-target species that are caught using these methods will be immediately returned to the habitat from which they were captured. For captured target species, they will be placed immediately into insulated coolers with a battery-operated aerator. Transport from the field site to licensed establishment will take up to 4 hours. Animals will experience aeration for the duration of transport, with water changes used as needed to maintain water quality and climate control in the vehicle to maintain temperature.

Once in the aquarium facility, animals will be exposed to varied biotic and abiotic factors that simulate natural variation that they would experience in the environment today, those conditions projected for the next century, and ecologically relevant parasite exposure. The animal's behavioural (e.g., sociality, escape response) and physiological traits (e.g., metabolic rate, locomotor performance) will be measured. When individual identification is necessary for the experimental design, individuals will be tagged with a minimally invasive method (i.e., visible implant elastomer) with an individually identifiable tag. These tags are administered subcutaneously and fluoresce under UV light, making them useful for behavioural studies involving real-time or video-recorded data collection. This tagging technique is commonly used on small-bodied fishes and has not been found to affect growth or survival even in small fishes at a larval ontogenetic stage (Hoey & McCormick 2006). Experiments will vary in duration, as animals will be acclimated to their experimental conditions for a period of weeks to months prior to testing behavioural or physiological traits. Animals may be tested for more than one procedure to look at correlations between behavioural and physiological traits. For example, we may test a fish

for both their fast start response (which quantifies their ability to escape a predator) and swimming performance in a swim tunnel (which tests a fish's swimming speed and behaviour under flow conditions that simulate natural flow in their native habitat) to determine how swimming performance influences their ability to effectively evade predators.

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

Any adverse effects are expected to be short term (typically seconds, minutes, or hours), including brief pain, minor weight loss, and changes in behaviour (e.g., activity, social interactions, use of shelter, feeding motivation). Any adverse effects will be monitored closely by the researchers and technicians, with careful documentation of any unexpected adverse effects. The parasite exposure protocol outlined here replicates the natural parasitism that these fish species already experience in natural habitats.

The environmental changes mimic those expected by the end of the century. All fish will be returned to conditions experienced today following experimentation and these treatments are not expected to cause any prolonged changes in the fish's health or welfare. The behavioural and physiological tests will all be short-term, and will not cause any prolonged changes in fish health.

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

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

The severity for most fish is expected to be mild (e.g., respirometry for measurement of metabolic rate). Similarly, manipulation of environmental conditions (e.g., temperature) is expected to be mild as well. Exposure to parasites is expected to have a mild severity and only simulate what the fish experiences naturally in the wild. A moderate severity will be the cumulative result from animals being used in more than one mild step in the protocol. We expect 70% of animals to experience a moderate severity and 30% of animals to experience a mild severity.

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

- Killed
- Rehomed

Replacement

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

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

As these studies objectives all center around studying the behaviour and physiology of fish, no alternative to live animals are possible. However, the magnitude of any pain or suffering will be minimal, as the protocols are not invasive and rely on studying the animal's traits in as natural a state as possible.

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

Previous modeling studies have assisted in generating hypotheses about the behaviour of live animals, which are used to design the protocols described here.

Why were they not suitable?

Apart of aiding in generating evidence-based hypotheses, these modeling studies cannot replace live animals for this work.

Reduction

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

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

The numbers of animals described above were determined using literature searches of the study topics, which provides us with an projected effect size and variability for the traits studies. I also have extensive experience in experimental design with the types of studies described here so have used prior experience to determine an appropriate sample size given the questions being asked.

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

I have used extensive literature searching to determine appropriate samples sizes given the questions asked. Typically sample sizes for individual experiments will vary from 200-500, which represents 10- 15 fish schools per treatment. We will use a power analysis for each study to ensure that we use the fewest fish possible to achieve a statistically significant outcome, should there be one. For example, using the R software environment for statistical computing and graphics (v4.3.1), I conducted a power analysis to determine the sample size necessary to have reasonable power to detect differences in metabolic rate using respirometry between individuals testing alone versus in a social context, which suggested that a sample size of 10 fish per treatment would give us ~95% power to detect a statistically significant effect if there is one.

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 a mixture of pilot studies (to ensure that experimental protocols are successful) and computer simulation/modelling studies to refine the experimental design prior executing the full 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.

Fish present ideal models to answer these questions, as they execute diverse and complex social behaviours that can be generalized to many other taxa. In addition, I have experience maintaining fishes successfully in a laboratory setting without any pain, suffering or distress. Further, the behavioural and physiological studies use minimally invasive procedures, that are sub-threshold or mild in severity, which will also minimize pain, suffering, distress, and lasting harm. A moderate threshold is only expected when fish are used in more than one mild behavioural or physiological assay.

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

These studies are specifically testing the effects of environmental change and parasites on fish behaviour and physiology, which cannot be replicated in less sentient animal.

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

I have over ten years' experience in the protocols described in this license, which I will use to provide training and oversight to the use of all in animals used. I will make refinements in terms of holding and handling to minimize welfare costs. I will ensure that all fish are held in tanks with enrichment in the form of a shelter, substrate, and, if appropriate, social partners to replicate the natural environment as much as possible during captivity. Holding conditions will be carefully controlled and monitored regularly, including temperature, light, and water quality. Second, handling of fish will be minimized to reduce stress, with air exposure minimized during collection and holding periods.

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

We will follow the experimental design and reporting guidelines published by Percie du Sert et al in 2020 in PLOS Biology (The ARRIVE guidelines 2.0) and Smith et al. 2018 in Laboratory Animals (The PREPARE guidelines). For behavioural assays, we will use the guidance from the ASAB Ethical Committee/ABS Animal Care Committee (2023 Animal Behaviour) for implementing the 3Rs. Killen et al. (2021 Journal of Experimental Biology) published a guideline for reporting metabolic rate data ascertained through aquatic intermittent flow respirometry, which we will use for studies involved respirometry data.

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

I will stay informed of changes in best practices for implementing the 3Rs through attendance at 3Rs workshops, reading of the scientific literature, and the mailing list with the Animals in Science Regulation Unit (with which we receive email updates). I also belong to multiple professional organizations and attend the annual meetings at which animal best practices are discussed, including the Society for Experimental Biology and the Fisheries Society of the British Isles.

3. Brain network changes in a rodent model of schizophrenia

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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, Animal behaviour, Electrophysiology, Schizophrenia, Psychedelics

Animal types	Life stages
Mice	adult
Rats	adult

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

To identify how the activity of brain networks and cognition are altered in a well-established rodent model of schizophrenia. The work will determine the ability of both standard and novel drugs to restore altered brain activity and behaviour to normal levels.

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

Why is it important to undertake this work?

Schizophrenia is a devastating brain disease that affects approximately 24 million people worldwide. It is frequently associated with significant personal distress and impairments in personal, family, social, educational, occupational and other important areas of life. People

with schizophrenia are 2 to 3 times more likely to die early than others in the general population (10 years on average) due to suicide and/or physical illnesses that include cardiovascular, metabolic and infectious diseases. Despite the acknowledged devastating effect of schizophrenia on so many sufferers, their families and other care- givers, the current range of drug treatments cannot improve all aspects of the disease and the severe side-effects of available drugs can lead to patients giving up their treatment.

In particular, current therapies for schizophrenia fail to improve patient symptoms such as memory, attention and decision-making deficits. These cognitive problems (symptoms that impact how we think, feel and act) are reported by patients as having the biggest negative impact on their quality of life. Indeed, these problems make it hard for sufferers to firstly find and then secondly hold down a job, further impacting their ability to re-join and make a positive contribution to society. Thus, there is a pressing need for research efforts to find new treatments for schizophrenia that target these devastating cognitive symptoms.

Our knowledge regarding changes in the brain that give rise to the symptoms of schizophrenia is still extremely limited, both from studies in patients and animal models. This project will address this gap by using a well-established rodent model of schizophrenia to reveal for the first time the activity changes in the brain that produce cognitive deficits. We will then use this knowledge to determine again for the first time the extent to which we can restore normal brain function with both commonly prescribed medications for schizophrenia and novel therapeutic strategies.

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

Outputs from this project will primarily be additions to academic knowledge. These outputs will be communicated as new information via: (a) poster and oral presentations at national and international scientific conferences; (b) publication of results in academic scientific Journals that are, wherever possible, open access to the public. Overall, our hope is that the project will greatly expand our knowledge regarding disease-relevant changes in brain activity that underpin schizophrenia and how new interventions may normalise such activity.

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

Short-term benefits will be the ongoing publication of new insights into how changes in brain activity underlie cognitive impairments in our model of schizophrenia. By the end of the project, we hope to have quantified how these deficits can be rectified by novel drug treatments such as psychedelics. Longer-term benefits will be to validate further our disease model of schizophrenia such that it can play an even more important role in new drug development. This will be underpinned by the project's novel identification of abnormal brain activity patterns as a new target that can be added to our existing disease-relevant anatomical and behavioural markers of schizophrenia. These we believe will be extremely attractive to pharmaceutical companies with an interest in developing new treatments for schizophrenia.

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

Our work fits within the wider research effort into treating schizophrenia. This will naturally encourage collaboration between our lab and others; for example, we can take tissue from our functional experiments and run further complementary analyses to correlate our findings with, for example, established changes in brain anatomy and neuronal markers of

disease. It is important to disseminate the knowledge gained from our work irrespective of whether our experimental outputs support or refute our initial experimental hypotheses. So, we will seek to publish all completed experiments, whether the approaches were successful or not, as reporting 'negative' findings to the field is as important as communicating 'positive' outcomes. To maximise the relevance of our laboratory animal work to patients in the clinic we will perform studies in both female and male animals. Our chosen disease model can be created in both male and female animals; however, it has been investigated and defined most thoroughly in female animals. Our strategy will, therefore, be to obtain new information first in females and then repeat those studies in males.

Species and numbers of animals expected to be used

- Mice: 2000
- Rats: 1000

Predicted harms

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

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

We choose to use rodents as many aspects of their behaviour and brain anatomy are fundamentally similar to humans. Our pre-existing knowledge of these aspects in rats and mice are also far greater than for any other species, including primates. Thus, rodents provide useful models of normal and, with suitable alteration, abnormal human brain and behaviour. We induce our rodent model of schizophrenia in adult animals and this reflects very well the specific symptoms and biological markers for the human condition. Whilst it is possible to induce similar changes in pre-term rodents, this can result in a less specific model for schizophrenia, rather, producing a more general model of abnormal brain development that reflects symptoms across many different diseases. Therefore, we feel our adult model provides a more specific representation of schizophrenia.

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

An animal will typically experience the following:

Schizophrenia model induction will be accomplished by injection of a drug (phencyclidine), typically by dosing every day for up to 10 days; control animals will be dosed similarly but receive only saline. We will then allow 7 days for phencyclidine to clear out from the animals' circulation. Behavioural testing will then test for the presence of a characteristic memory deficit in model animals.

Either:
behavioural testing to probe multiple types of memory and decision making. Drugs may be used (typically by injection or oral dosing) to determine their capacity to modify or restore behavioural deficits; or implantation of recording and stimulating devices into target areas of the brain under anaesthesia These will measure functional brain activity/connectivity during the performance of behavioural tasks. Drugs may be used (typically by injection or oral dosing) to determine their capacity to modify or restore behaviour and functional brain activity; or we will test for the benefits of non-pharmacological ‘treatment’ by allowing

animals to exercise, typically by running in wheels. Similar aerobic exercise is beneficial in human patients, so has the potential to reduce or replace their need for drugs, but we do not know how aerobic exercise exerts these improvements in brain function and behaviour.

Experiments will typically take up to 3 months but some will take up to 10 months in order to determine how long behavioural and brain activity changes persist. Some of our behavioural tasks also take weeks to months for animals to learn. We will in parallel also test for positive response to drugs over this extended period.

Psychedelic drugs. A particular treatment focus in this project will be on psychedelic drugs. These compounds have been shown in recent human clinical studies to be highly effective for a range of cognitive and neuropsychological disorders (such as major depressive disorder) that are difficult or impossible to treat in many people with currently prescribed drugs. Whilst psychedelic drugs produce positive changes in the behaviour of patients, often after only a single treatment, we have little understanding of how they change brain activity to produce this behavioural improvement. Thus, we will test psychedelics as a priority in our project to expand our knowledge of their mechanism of action in the brain and their ability to treat cognitive symptoms in our animal model for schizophrenia. To determine whether psychedelics out-perform current antipsychotics we will compare their effectiveness against existing 'best available' human therapies. Permission to use restricted substances: Psychedelics and phencyclidine are controlled drugs in the UK and their use falls within the Misuse of Drugs Act (1971). In order to use these drugs in this project we have obtained all appropriate licensing and permission from the UK Home Office for that use in our research.

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

Our model induction process using phencyclidine does impact on the behaviour of the animal for a short time after every dose. Thus, animals can appear slightly confused and unaware of their surroundings. However, this passes quickly (less than 1 hour) and the duration and intensity of these drug effects reduces quickly after each dose as the animal develops tolerance. Once established, model animals are virtually identical to controls in terms of their general behaviour - it is only when given cognitive tests or drugs that differences can be seen in model animals.

Some behavioural tests will require food restriction of the animal. This is because in these tests we use food to encourage behaviour during training and testing - so animals receive food for correct performance during the task. Animals are expected to lose some weight during food restriction periods and this will be monitored very closely. If their weight loss approaches 15% compared to free-feeding animals, we will reinstate free feeding to shift their weight towards the optimal (around 10%) reduction figure compared to controls; we expect such weight gain to occur within 24-48hrs of return to free- feeding. We will only restrict food immediately prior to and on behavioural testing days; on all other days, animals will have free access to food and will always have free access to water.

Recovery following surgery will result in some pain for the animal as it recovers from the anaesthetic but this will be controlled by pain-reducing drugs and animals will be monitored closely during their recovery. During recording after the recovery period, the animals will have to carry small recording and stimulating devices on their head, but these are very light so only have a minimal impact on the natural behaviour of the animal. The latter will be aided by prioritising recordings where measurements are transmitted wirelessly from these head-mounted devices so that there is also no requirement to

connect the animal via wires to the equipment in these cases.

Drugs used to modify behaviour and brain activity are of course likely to affect cognition, but we will minimise the chance of any serious adverse effects by selecting doses from the literature that are known to be safe. In cases where that evidence is not available for selected compounds, or we have not used them before, we will perform a pilot experiment in a small group of animals where we test intended experimental doses of that drug incrementally (one dose per animal) and monitor adverse effects over 1 hour. We will monitor the lowest dose first for adverse effects before progressing to the next highest dose. We do not expect adverse effects from dosing but if these do arise and last for 1 hour the animal will be killed humanely 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)?

Moderate severity: Animals that experience model induction via injections over several days of drug/vehicle (or are drug naive) followed by behaviour with/without further drug challenge - 70% of animals

Animals that experience model induction via injections over several days of drug/vehicle (or are drug naive) followed by brain activity recording under anaesthesia - 15% of animals

(ii) Animals that experience model induction via injections over several days (or are drug naive), then experience electrode implantation followed by brain activity recording during behavioural tasks with/without further drug challenge - 10% 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?

It is impossible to mimic brain and behaviour interactions in cell systems, so studies using live animals are vital to obtain a greater understanding of normal and abnormal mental states and to test the effectiveness of new drugs. Some *in vitro* approaches, however, may provide valuable tools to screen potential treatment compounds prior to their use in animals.

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

Computational models, in vitro preparations.

Why were they not suitable?

This work must use whole animals, as understanding behaviour and the required brain activity to produce that behaviour is a central feature of the project. This cannot be studied effectively by using reduced in vitro preparations, and computational approaches lack the required complexity due to insufficient biological data. To date, there is no suitable alternative to the use of rodents for behavioural studies that do not involve human subjects and we are still extremely limited in our ability to measure neural activity directly from the human 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?

Behaviour/behavioural pharmacology studies. Our general expectation for animal numbers is that we will require 10 per group for behaviour. This is based on data acquired by us previously where we have seen robust outcomes using this cohort size. We will monitor closely for any changes in our data that may support an increase or decrease in required sample sizes, for example, to show a significant effect of drug treatment. Where there is limited or no previous relevant information for a specific experimental design, we will compute and predict sample sizes that are likely to be sufficient to provide a robust experimental result based on previous most relevant experiments from ourselves or other groups. Data analyses will be carried out according to a pre-specified statistical analysis plan drawn up in conjunction with professional statistician experts.

Studies recording brain activity. Numbers required for these depend on the question and technique at hand and, most importantly, the size of the effect in our experimental animals. Similar studies in the literature typically require a sample size of approximately 8-10/group and this matches well with our estimated group size of 10 to see drug treatment effects. Studies recording brain activity are usually carried out by accumulating recordings from one or two animals at a time. This sequential use of animals will allow us in many cases to make ‘waypoint’ checks well before we reach our predicted required group sizes. This will ensure that we are not using too many animals, or that we need to add more animals to achieve our scientific goals. For example, if we predicted that a group size of 10 animals would be required to effectively test our hypothesis, we can double check ‘halfway’ through an experiment, for example, once we have 5 animals in each group, whether we will still need that total of 10 animals per group.

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

Experimental design. We will use experiments with several variables, where we predict that those variables will have some effect on our experimental measures (e.g., improvement in memory). These variables include the type of animal (e.g., control or disease model groups) and treatment (e.g., high or low doses of a particular drug or periods of exercise with short or long duration). Such designs allow us to test whether each variable has an influence on its own and whether together they have a combined effect (e.g., changing the dose of a drug may produce different behavioural changes in model

versus control animals). By combining several variables in this way, we increase our knowledge about effects in our model animals whilst keeping the number of animals and experiments required to a minimum.

The quality of our measurements will be maximised by good training of researchers. To reduce the chances of experimenter handling affecting results, we will use tasks wherever possible in which animals can learn and perform at their own pace, that is, without repeated handling by the experimenter. To make our testing as fair as possible we will: randomly allocate animals to treatment groups; minimise the chances that we get a particular result simply due to the order in which animals experience experimental stages; and where possible ensure that experimenters are unaware of whether animals are in a particular treatment group until all data capture and analysis stages have been completed. To maximise the quality of our results we will adhere to national standards ("ARRIVE" and “PREPARE” guidelines) when planning and carrying out our research and publishing our findings.

Whilst most animals will experience either no drug or a single drug exposure, we require some animals to experience more than one drug/compound to produce a change in behaviour. In cases of repeated challenge this will be for cases where we could not substitute a naive animal. This approach will also reduce the number of animals required to meet our Objectives.

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

When testing a drug that is new to the laboratory, we will first conduct a pilot experiment using a small group of naïve animals. Experimental doses will be given incrementally (one dose per animal) and we will monitor for adverse effects over 1 hour. We will test the lowest dose first and if there is no adverse effect progress to the next highest dose in the next animal. This procedure will provide a safe dose range for our experimental groups.

Refinement

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

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.
We will use mouse and rat species as these are of low neurophysiological sensitivity, yet their brains show good anatomical and functional similarities to those in the human brain. All procedures in this license range from non-recovery to moderate severity.

Our chosen animal model for schizophrenia develops both behavioural/cognitive deficits and abnormal changes in the brain that mimic very well those seen in schizophrenic patients. Importantly, for model animals their overall general behaviour is unaffected and deficits are only observable when you test for them. Thus, our model induction produces no observable long-term pain, suffering or distress.
Our subsequent behavioural measures will often require animals to simply behave

spontaneously; for example, we will measure whether they can detect a change in their environment that requires prior memory of, for example, the type of objects encountered previously. Some animals will be trained to perform a task, which will require food restriction on training and testing days as we use food to reward learning and correct task performance. We do not expect significant adverse effects from this food restriction but will monitor the weights of these animals very closely. If we detect substantial weight loss than cannot be reversed by full food provision the animal will be killed humanely immediately.

In experiments where we record brain activity from animals during behaviour, we will use well- established techniques developed over the last 60 years. This requires animals to undergo recovery surgery under general anaesthesia where we will minimise the chances of infection and peri-operative analgesia to reduce pain. Animals will be monitored closely post-operatively for several days for signs of pain and further analgesia will be provided if required. These procedures are now well established in our labs.

In experiments where we dose animals with drugs we will use doses, routes and volumes that are suitable for the species. We will make sure that any novel compounds are tested in a small group of animals first to ensure safety and tolerance before dosing our experimental groups. All drugs are intended to improve or normalise cognitive performance of animals.

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

Our experiments require measurements of behaviour and cognition in adult rodents to model disease- relevant changes in the human adult population. Rats and mice are relatively simple species when compared to human but they exhibit similar cognition and brain anatomy. It is not possible to use lower species to reproduce the same range of human behaviour and comparative brain anatomy and function.

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

As described above, we will monitor general physiology and behaviour of our animals closely both during and outside experimental phases. For recovery surgery procedures we will apply appropriate post-operative care and analgesia. We will adjust our procedures if required through our close monitoring of animals at all stages and seek advice from NVS and other animal unit staff as appropriate.

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

We will follow the standards set by PREPARE (experiment preparation and execution), LASA (aseptic surgery), NC3Rs (e.g., blood sampling) and the Joint Working Group on refinement (Morton et al., 2001 Lab Animal 35).

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

Training routes and updates are provided by our animal house staff regularly and recent advances in animal husbandry and care are communicated effectively to all users. All activities under this license will also be informed by the regular NC3Rs newsletter. Laboratory workers are normally members of established national (e.g., British Neuroscience Association) and international bodies (e.g., International Brain Research

Organisation) that are active in promoting best practice for animal research.

4. Cerebellar contributions to motor and non- motor behaviour

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

Cerebellum, neuron, emotion, skilled movement, coordination

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

To advance understanding of how neural processing within the cerebellum and its interconnections with the rest of the nervous system controls behaviour, including higher brain functions of cognition and emotion. To achieve our objectives the experiments will include use of genetically altered animals that enable specific neural pathways and/or brain regions of the cerebellum and related circuits to be targeted.

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

Why is it important to undertake this work?

Understanding how the brain controls behaviour is one of the fundamental questions in science. The cerebellum is the largest sensorimotor structure in the brain, accounting for around 80% of all neurons. Its role in the coordination of voluntary and reflex movements is well known, and defects in this region underly a range of disease characterised by movement disorders including spinocerebellar ataxias and essential tremor. However, functional imaging work in humans indicates that the cerebellum is also engaged in the higher brain functions of cognition and emotions. The mechanism underlying these higher functions is unknown and the primary aim of the outlined work is to identify the neuronal networks through which the cerebellum influences higher brain functions. The work is important as a number of serious personality disorders are known to be linked to cerebellar dysfunction, including schizophrenia and generalised anxiety disorder. By advancing understanding it is to be hoped that, in due course, improved treatments for such conditions will be identified.

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

The outlined studies will generate data relevant to advancing understanding of the neural processes that underpin behaviour. Specifically, the project will explore and test hypotheses concerning the brain regions responsible for: 1) the encoding and retrieval of different types of memory information; and 2) the neural pathways recruited during specific types of behaviour. The findings of the study will form the basis of publications in peer-reviewed scientific journals and will be presented at national and international scientific conferences and meetings.

Since the plan of work seeks to understand fundamental aspects of nervous system functioning it is not anticipated that there will be direct clinical benefits. Instead, in the short to medium term, the findings will assist the development of computational models of sensorimotor circuits and aid the interpretation of functional imaging in humans.

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

The direct beneficiaries of the outlined work will be researchers in the fields of sensorimotor, behavioural, cognitive and computational neuroscience. The outcomes of this work will generate insights and findings that will advance the field. The outcomes will be published and shared through scientific journals and online data repositories.

These benefits will be achieved in the short to medium term (3-5 years). In the longer term (5-10 years), it is to be hoped that by advancing knowledge in this field, the work will contribute to the identification of improved treatments for patients suffering from a range of conditions involving cerebellar defects, such as spinocerebellar ataxias and essential tremor and generalised anxiety disorder. For example, understanding how components of a fearful stimulus are detected and encoded in the brain is fundamental to advancing knowledge of fear learning and memory mechanisms in order to provide a rational approach to new treatments for psychological disorders such as post-traumatic stress disorder (PTSD) and generalised anxiety disorder. In particular, the project will shed light on the neural mechanisms that underlie fear extinction learning and memory - processes which are thought to be important in common forms of cognitive-behavioural therapy.

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

The findings of the study will be published in peer-reviewed scientific journals and presented at national and international scientific conferences and meetings.

Species and numbers of animals expected to be used

- Rats: 400
- 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 the findings of the proposed experiments to be relevant to understanding the functioning of the human brain, the study requires an animal with a brain that is anatomically and physiologically similar to that of humans. Rats and mice are the least sentient species that meet the required criteria. Where possible, adult animals will be used as it is important to use an animal with a fully developed brain for these studies. Rats will be required for studies aimed at assessing the influence of the cerebellum on cognition and emotion, since unlike mice, they are able to reliably learn and perform the complex behavioural tasks required to undertake these studies. Also, their size allows accommodation of the implants required to address most of our objectives. Mice will be used for studies in which genetic manipulation of the animal is required in order to target specific neural pathways and/or regions of the cerebellum as genetically altered mouse lines suitable for these studies are readily available. Mice will also be used for studies requiring the position of the animal's head to be temporarily fixed, to enable precise recordings of brain activity to be made, as they can be easily trained to accept being restrained in this way.

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

Upon arrival in the unit, the animals will be habituated to human contact by regular handling and hand feeding treats. The majority of animals will undergo a surgical procedure, performed under general anaesthesia to allow injection of neuroactive substances, neuroanatomical tracers and viral vectors. The latter will have one or more cannulae implanted into the brain to allow subsequent infusion of agents to manipulate neural pathway activity. Animals may also have in addition or instead, electrodes, optic probes or a miniscope implanted into the brain and in some animals, electrodes into the muscles of the limbs, neck, chest or back. Surgery will be performed using strict aseptic precautions and all animals will be given pain killers post surgery, which will be maintained until the animals are showing no overt signs of pain. All animals are expected to make an uneventful recovery from surgery and to resume normal behaviour within a few hours of recovery. Once the surgical wound has healed, some animals (mainly rats) will undergo a range of behavioural tests to assess motor, cognitive and emotional responses. Prior to testing, the animals may be given drugs that temporarily modulate neuronal function via implanted cannulae. During testing, recording of brain activity may be made. For studies of brain circuits involved in affective behaviour some rats may undergo Pavlovian fear conditioning involving a conditioned stimulus (eg an auditory tone) combined with an unconditioned stimulus (a footshock). In order to motivate the animals to engage with tasks involving food rewards, they may be maintained on a restricted diet or, in the case of mice being used in head fixation studies, restricted water intake. Throughout the study period, all animals are expected to remain in good health and to continue to behave normally. At the end of the study the animals will be killed humanely so that the brain can

be removed for analysis.

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

Animals undergoing surgery are expected to experience mild transitory distress, lasting about a minute, during the induction of anaesthesia. Following surgery, all animals are expected to make an uneventful recovery. Upon recovery, animals are expected to experience some pain, which will be mitigated by the use of pain killers. Pain control measures will be maintained until the animal is no longer showing any overt signs of pain. In order to motivate animals to engage in tasks that involve a food reward, the animals may be placed on a restricted diet and therefore may experience mild hunger. Water restriction in mice will be limited to motivating behaviour and will not result in any lasting harm.

Aversive paradigms will be restricted to studies in rats only. In laboratory settings, Pavlovian cued fear conditioning is the most widely used paradigm to study neural circuits that underpin survival behaviours associated with fear learning and memory. The advantages of this paradigm are that it involves well described learning and memory processes (which include acquisition, recall, extinction and spontaneous recovery phases) that can be monitored in rats through well-defined behavioural outputs e.g. changes in freezing behaviour, autonomic responses and ultrasonic vocalizations. One of the key objectives of the project is to study the neural correlates of fear learning and memory, particularly during extinction of a conditioned response. Pavlovian cued fear conditioning relies on well controlled sensory stimuli - an aversive unconditioned stimulus (US) and a neutral conditioned stimulus (CS), that provide precise (ms resolution) event markers to be able to correlate neural activity with behavioural readouts of fear conditioning.

Fear conditioning does not result in any lasting harm. At the end of the study, all animals will be killed humanely, in order that their brain can be removed for analysis.
Expected severity categories and the proportion of animals in each category, per species.

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

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?

Investigate how neural networks within the brain alter, in response to an animal’s lived experience, to modulate behaviour; can only be achieved using a fully conscious animal.

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

Functional imaging studies in human subjects.
Computer simulations.

Why were they not suitable?

The temporal and spatial resolution of non-invasive functional imaging studies is insufficient to obtain the kind of information we seek to provide.

For computer simulations to be realistic and productive of testable hypotheses the modelling must be based on the kind of information we seek to provide.

Reduction

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

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

The estimate for the number of animals required to complete the study has been determined by extrapolation of data derived from previous related studies undertaken by my research group.

Behavioural experiments normally require n=20 animals per group. We typically carry out 3-4 behavioural tasks on an experimental group. Electrophysiological studies typically require a minimum of n=50 neurons from approximately n=25 animals (some animals provide a higher yield than others due to unpredictable differences between animals in the stability of recording). The number of animals include control groups. Animals will be randomized to experimental groups as will the order of experimental intervention. Experimenters will be blinded to the drug and virus type.

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

The experimental design for individual studies will be undertaken with the assistance of my institutes dedicated biostatistician and checked using the NC3R's Experimental Design Assistant tool. Wherever possible, animals will act as their own control (e.g. when studying temporary interventions of drugs on neural activity we compare the effects of vehicle and drug in the same animal). This increases power by pairing in addition to reducing animal numbers. Animals may be tested on multiple behavioural tasks, aiding interpretation of data and minimising the total number of animals required to meet our objectives.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?
The use of multi-electrode technology allows us to record from multiple neurons simultaneously, while miniscopes allow multiple neurons to be imaged at the same time over the course of many days, thereby significantly increasing our yield per animal and reducing the overall numbers required to meet our objectives. In anatomical tracing experiments the experimental unit is individual injection sites.

When unilateral paths are studied, economy of animals will be achieved by making injections of tracer on both sides of the brain. For studies in which viral tracers are used, controls are also performed, whenever practicable, in the same animal.

Refinement

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

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

The outlined studies will be undertaken using adult rats and mice. Upon arrival in the unit the animals will be acclimatised and habituated to humans by regular handling and feeding food treats. Most animals will undergo a surgical procedure, performed under general anaesthesia and using strict aseptic precautions. All animals will be given pain killers post-surgery, which will be maintained until no overt signs of pain are detectable. Once the surgical wound has healed, the animals will undergo behavioural testing. To motivate them to take part in tasks involving a food or water reward, some animals may be placed on a restricted food or water intake (mice for the latter). Food or water restriction will be limited to the minimum required to motivate the animals to perform the task and will not result in any adverse effects other than mild hunger or thirst. The vast majority of behavioural tests used are non-aversive however, some animals (rats only) will be tested in aversive tests (e.g. auditory cued Pavlovian conditioning) that cause transient pain or distress but do not result in any lasting harm. Some animals will be given drugs via implanted cannulae, prior to behavioural testing but these are not expected to have any adverse effects. Similarly, methods to selectively modulate specific brain pathways temporarily during behaviour are not expected to have any adverse effects. Experiments where a high level of neural recording stability is required will be restricted to mice because they tolerate head fixation. At the end of the study, all animals will be killed humanely so that their brains can be recovered post mortem for analysis.

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

It is not feasible to conduct the studies in neonate animals due to the size and complexity of the implants involved and because the brain pathways we seek to understand are not fully developed.

Non-mammalian species are unsuitable as they either lack a cerebellum (arthropods) or the cerebellar- like structures in animals such as teleosts (e.g. zebrafish) lack the connectivity with higher centres we seek to understand.

Although cerebellar activity can be studied in anaesthetized or decerebrate animals, activity in cerebro- cerebellar pathways is abnormal or absent, and study of cerebellar interactions with higher order centres is correspondingly limited or not possible. These pathways need to be intact for the behavioural readouts central to the hypotheses being tested.

Ex vivo brain slice preparations lack the connectivity of the intact brain essential for a full understanding of the neural networks we seek to understand. However, brain slices will be used wherever our objectives are not affected by such limitations.

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

The procedures conducted under this licence have all been extensively refined by my group, during earlier related studies, to minimise the welfare consequences on the animals. For example, where appropriate we now use silicon arrays for recording single unit neural activity. In some brain sites the yield of neurons is far higher than we were previously able to achieve with other types of recording electrode. We have also developed custom-designed microdrives and used 3D print technology to make them, reducing the size and weight of head implants. The bespoke design means we can incorporate multiple microodrives and record from more than one brain region simultaneously, increasing data yield per animal. Going forward, if grant funding permits, we also aim to replace tethered with wireless recording.

An additional refinement under our current PPL concerns the stimulus parameters to study fear conditioned extinction phenotypes in outbred rat strains. Previous studies have used 2 sec footshocks (e.g. Ji et al, 2018; Presto et al, 2021). We found that a 1 second duration is sufficient to produce reliable conditioned freezing behaviour.
Prior to the start of every behavioural study, the animals will be habituated to human contact by regular handling. Some animals (rats only) will be tested in aversive tests (e.g. auditory cued fear conditioning using footshock) that cause transient pain or distress but do not result in any lasting harm. However the majority of behavioural tests used are non-aversive and do not cause any harm. When food restriction is required, the animals are monitored by regular weighing and weight loss is restricted to a maximum of 15% of body weight. In addition, highly palatable food rewards are used for these studies which further reduces the need for food restriction. Water restriction is used in mice to motivate them to perform behavioural tasks. The degree of food or water restriction used will be the minimum required to motivate the animal on the rewarded task. Restrictions will be adjusted throughout the study to the minimum at each training stage. We will use an automated system developed in collaboration with NC3Rs which will enable us to precisely schedule and monitor fluid consumption. We will also follow the guidance and recommendations from the NC3Rs on fluid control and head fixation in mice. The surgical procedures are all performed by experienced surgeons and are undertaken in-line with LASA guidelines. Post-surgery, all animals are given pain killers until they are no longer showing any overt signs of suffering. The electrophysiological and imaging studies conducted post-surgery have no impact on the well-being of the animals. At the end of the study all animals are killed humanely under terminal anaesthesia.

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

The NC3Rs Experimental Design Assistant, ARRIVE 2.0 guidelines for design and reporting, institutional SOPs for handling and injections, LASA guidelines for aseptic surgery, comprehensive and contemporary review of published papers in the field.

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

I receive regular 3Rs newsletters from our local and regional NC3Rs champions and

participate in my institutional 3Rs events. In addition, I monitor advances in best practice by regularly attending conferences in the field, liaising with national and international collaborators, and through weekly journal clubs appraising recent publications. I also maintain active collaborations with engineers and technology developers in the field, optimising opportunities to pilot and/or implement the most efficient and minimally invasive methods of neurophysiological data collection.

5. Circuit and behaviour dysfunction in rodent models of neurodevelopmental 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

Neurodevelopmental disorders, therapies, neuron, behaviour, cognition

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

To understand the biological mechanisms underlying neurodevelopmental disorders (NDDs) with the aim of translating this knowledge into effective therapies.

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

Why is it important to undertake this work?

NDDs are caused by altered brain maturation due to genetic and environmental factors. Developmental brain disorders can be severely limiting, and there is an increasing

diagnosis of NDDs in the UK population.

Despite decades of research, current treatments for these disorders focus on managing symptoms and on behavioural therapy rather than on acting on the underlying cause. The unmet need, therefore, is to develop ways to more effectively treat NDDs. Rodent models of NDDs enable studies of the causes underlying these disorders and support their research. However, a major obstacle for NDD drug development is the lack of robust measures in these models that can predict clinical success.

The overarching aim of this project is to address this challenge by using multiple established rodent models of NDDs that are of interest because of their similarity to corresponding human disorders to identify robust, clinically relevant measures that will allow the rigorous testing of potential therapeutic interventions for NDDs.

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

The experiments in this project will provide valuable insight into the underlying causes of the cognitive and behavioural symptoms associated with developmental brain disorders. Furthermore, comparison between different rodent models of NDDs will determine whether different genetic disruptions lead to common disease states. Our experiments will also address the duration and timing of treatment in the appearance and improvement of brain-based deficits and the clinically-relevant behaviours that they lead to.

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

The outputs of this research will directly and immediately benefit the global research community studying the biological basis of behaviour and cognition and how this differs in developmental brain disorders. The methods developed in this work will also have potential applications and benefits in the study of other childhood disorders and psychiatric diseases.

In the medium term, our findings may eventually help the classification of NDDs both in terms of their progression as well as their amenability to treatment across the lifespan; this would represent a major advance for the global research community and for people with lived experience of NDDs since current treatment methods focus only on clinical or genetic criteria.

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

We aim to share the results from this work in the following ways:

- communication with members of the scientific community through collaborations and at relevant conferences
- publication of our work in open access research journals
- making completed datasets available to the scientific community
- engaging in public engagement through family groups, patients, charities and social media.

Species and numbers of animals expected to be used

- Mice: 1500
- Rats: 20000

Predicted harms

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

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

The project will investigate the functioning of brain cells within the intact brain, and how brain alterations relate to the cognitive and behavioural changes we are assessing in rodent models of NDDs. To this end, this project will assess rats and mice from early life to late adulthood since hallmarks of developmental disorders emerge during childhood and usually persist over the lifespan. We use these animals as models for several reasons:

The cognitive functions and behaviours related to NDDs we aim to understand, as well as their underlying brain circuits, are generally well conserved across mammalian species. While details may differ in humans, the general circuits and mechanisms are likely to be similar. By making advances first in rodents, it is likely to be easier to address whether similar mechanisms arise in the human brain.

- It is not possible to use simpler established models (e.g. flies or worms) because these species do not use the same cognitive functions and underlying neural circuitry.

- Many experimental tools already exist to support neuroscience experiments in rats and mice; this enables more rapid progress than would be possible with other similar species.

- Several rat and mouse models of known genetic causes of NDDs have been developed; we will use these to understand how brain alterations relate to the cognitive and behavioural changes associated with NDDs. Even though rats and mice resemble each other, they are estimated to have separated in evolution more than 12 million years ago, making them less similar to each other than humans are to chimpanzees. Cross-species validation utilizing both rats and mice will provide a more comprehensive understanding of NDDs and enhances the translatability of findings from basic science to human contexts.

- The most appropriate model is chosen for every experiment since one species (rat or mouse) sometimes confers a distinct experimental advantage over the other. For example, many molecular and genetic tools exist in mice that are not available in rats. On the other hand, rats have a rich behavioural repertoire; they adopt flexible approaches to novel situations and have extensive social interactions including cooperativity, two cognitive and behavioural domains commonly affected in NDDs.

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

The project will typically involve two different types of experiment:

To study how cognition and behaviour develops during juvenile development to adulthood, and how it is affected in rodent models of NDDs, animals will be trained and tested on one or more behavioural paradigms over a period of 1 week to 3 months. In some studies, they will be administered compounds (in their food or via injections) to test whether these can correct or prevent the emergence of any cognitive/behavioural differences. At the end of

the experiment, the animals will be killed humanely, and brain tissue may be taken for further analysis.

To investigate information processing by neurons that underpins cognition and behaviour, and how this differs in rodent models of NDDs, a typical experiment will involve recording and/or manipulation of the activity of neurons as animals perform cognitive tests. The recordings of neural activity will tell us what cognitive processes the neuron represents. The manipulations will enable us to test contributions of neurons to cognitive and behavioural performance. In some of these studies, we will map connections within and between different brain regions using functional magnetic resonance imaging (MRI). In other studies, we will monitor sleep and wake states using electroencephalographic (EEG) recordings. And in other studies we will implant electrodes into different locations of the brain to record how cells respond to a variety of situations, for example social interactions or exploration of a new environment. Most of these experiments require surgical procedures to attach devices to the head or implant devices into the animal's brain to image, record, or manipulate neural activity. In some cases, another surgery is required to inject substances into the brain to alter neuronal function. All of these surgical procedures will be carried out under general anaesthesia and analgesia, and typically last between 1 and 4 hours. At the end of the experiment, the animals will be killed humanely, and brain tissue may be taken for further analysis.

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

Rodent models of NDDs may show abnormal adaptive behaviour throughout the lifespan. These are not usually obvious and require close inspection to identify subtle differences from normal rodents.

The major expected impacts arise from experiments requiring surgery to inject substances into the brain and to attach/implant devices for recording from the brain. These may include transient pain and weight loss, which will be mitigated with pain relieving medication and supportive care. These procedures are carried out with refined surgical protocols and are not typically associated with other adverse effects. They are not expected to cause more than minor and transient distress or suffering to the animals.

Animals may be housed singly where there is a concern of aggressive behaviour or increased likelihood of damage to attached/implanted devices, for example for electrophysiology or optical experiments. Single housing is thought to be stressful to rodents and will only occur when scientifically justified. In such cases, the duration will be limited to the minimum period necessary, and all cages will be enriched with objects for exploration and play. These methods have been shown to reduce stress in rodents.

Expected severity categories and the 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 not expected to be classified above a moderate level. This applies to all animals used for the project.

We expect that for 30-40% of animals the severity will be mild or subthreshold since they will be used only used for breeding, behavioural testing and/or administration of

compounds followed by humane killing for tissues. The remaining 60-70% will experience moderate suffering as they will have surgical procedures as described above and or be tested on behaviours that may reach moderate severity.

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

- Killed
- Rehomed
- 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 goal of this project is to understand how changes in the function of cells in the intact brain relate to altered behaviour and physiology in rodent models of NDDs. To date, experimental systems that allow this are limited and must involve the use of vertebrate animals. While reduced in vitro preparations such as cell line and neuron cultures can be used to assess certain aspects of cellular function, these do not faithfully reflect the chemical and electrical properties of cells in intact networks of the brain and how these communicate to instruct behaviour. We have chosen to use rats and mice for these studies as they are the lowest order mammal in which these aspects of brain function and behaviour can be reproducibly assessed while also having the ability to perform genetic and environmental manipulations that cause NDDs in humans. Since these rodent models mimic key features of NDDs in humans, they allow us to disentangle processes and factors contributing to the underlying pathology and can be used to develop treatment strategies.

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

Human brain imaging studies Cell culture systems Computational models

Why were they not suitable?

Human brain imaging studies are useful as part of an overall refined approach, and one that we are using. However, while functional brain imaging in humans can identify brain areas and circuits that may be involved in cognitive processes, including those that may be affected in brain disorders, they have three main weaknesses. First, to ensure reliability in MRI studies, sufficiently large numbers of subjects need to be included; despite the prevalence of NDDs in the general population, it is a challenge to recruit enough individuals affected by these complex conditions. Second, MRI cannot distinguish between excitatory and inhibitory signals or different cell types. Third, while it is possible to identify which brain regions are active during specific cognitive processes, MRI studies cannot determine whether that brain activity causes or is required for those processes. Animal models allow for direct recording of neural activity, identification of specific cells in specific circuits, and the ability to make interventions that address causal questions.

Cell culture systems do not have the organisation of real brains, do not process sensory

inputs and do not execute behaviours. They therefore cannot be used as alternatives to answer the questions we aim to address.

Computational models are useful as part of an overall refined approach, and can be used to simulate aspects of brain function and to make predictions about how biological substrates can implement cognitive functions. However, it is not possible to use them as alternatives to answer the questions we aim to address because they don't yet explain the cognitive functions we wish to understand.

Development of better models requires data that can only be obtained with experiments in which animals use the cognitive functions we aim to understand.

Reduction

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

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

Statistical analyses have been used to estimate how many animals will be needed per experiment. These analyses are based on previous experience with these types of studies and expectations of an expanding number of researchers and projects over the next five years.

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

It is essential that the number of animals we use allows us to collect data that are robust. We have many years of experience in performing experiments using animals and this allows us to use specific statistical methods to predict how big an effect we can anticipate and how variable that effect will be. Using this information, we can estimate the numbers of experiments we need to carry out. Good experimental design means that we assess drug effects alongside non-drug treatments or control animals with those obtained from genetically modified animals.

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. Before performing experiments involving surgeries, a small number of animals will be used to confirm and test that the surgical procedure yields the desired scientific outcome (e.g. stereotaxic coordinates, expression of transgenes from viral vectors).

Sharing tissue. Wherever possible, multiple measurements will be taken in the same animal over time, across the lifespan, or before and after treatment, to increase the statistical power of our datasets. When possible, experimenters are also encouraged to share tissue from the same animal which will reduce the overall number of animals required for experimentation.

Refinement

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

Which animal models and methods 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 altered rats and mice as a model system. Both species are genetically very similar to humans and have anatomical organisation of brain circuits underlying behaviour and cognition that are similar to humans (unlike invertebrate or other non-mammalian model systems). Since this project aims to understand the functioning of brain cells within intact circuits in the brain, and how brain changes relate to the behavioural differences in rodent models of NDDs, common anatomical organisation of the brain is crucial, as is the ability to exhibit a range of cognitive abilities and behaviours exhibited by people affected by NDDs such as everyday memory, behavioural flexibility, and social behaviours and cognition.

Rats: The rat is the species of choice for most of the experiments in the proposed programme of work. We use rats rather than mice because they have a richer behavioural repertoire than mice; this includes extensive social interactions and adopting flexible approaches to novel situations, two domains specifically affected in NDDs such as autism.

Mice: In a subset of experiments, we will use mice instead of rats. Although their behavioural repertoire is not as strong as rats with regards to the cognitive and social domains relevant to NDDs, more molecular tools are established in mice; this makes them particularly amenable to genetic and molecular manipulations that provide refined approaches to manipulate specific neurons and brain circuits.

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

The project will investigate the functioning of brain cells within the intact brain, and how brain changes relate to the behavioural changes we are assessing in rodent models of NDDs. We have chosen to use rats and mice for these studies as they are the lowest order mammal in which these aspects of brain function and behaviour can be reproducibly assessed while also having the ability to perform genetic and environmental manipulations that cause NDDs in humans. Since these animal models mimic many human features of NDDs, they allow us to disentangle processes and factors contributing to the underlying pathology and can be used to develop treatment strategies.

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

We will adopt several refinements that minimise welfare costs, which by increasing the precision of experimental tests and manipulations will lead to clearer and increasingly specific answers to the questions we aim to address:

Animals will be housed in groups and cages will be enriched with tubes and objects for

exploration to reduce stress and promote healthy social interaction. In all our experiments we are mindful of the need for refinement to reduce suffering, and appropriate modifications to protocols are incorporated where possible. Protocols will be carried out in the most humane way possible. All surgery will be performed under general anaesthesia, using aseptic technique and pain relief will be administered during recovery to minimise distress. Where possible, protocols to monitor brain activity in freely moving animals will make use of wireless transmitters to reduce distress that may occur from cables limiting movement. The animals in these studies will be cared for by trained staff within a well-resourced and well-equipped modern animal facility that maintains specific pathogen-free status/health; they will be carefully monitored to ensure that suffering greater than minor and transient in their home environment does not occur.

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

We will follow all best practice guidance provided by our institution. We will also follow procedures that we and others have published that establish refined methods for the specific experimental questions we aim to address.

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

We have excellent institutional support and training to stay informed about and implement advances in the 3Rs. The Home Office Liaison Contact (HOLC) circulates HO guidance notes and welfare organizations' newsletters through emails. The Named Veterinary Surgeon (NVS) team and full time Named Training and Competency Officer (NTCO) work together with PIs to refine procedures and disseminate best practice ideas.

6. Damage and repair of musculoskeletal tissues

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

Cartilage, Arthritis, Regeneration, Osteoarthritis, Regenerative medicine

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The scope of this study is to understand the molecular mechanisms of tissue damage and reparative processes in musculoskeletal diseases, with the final aim of identifying and validating novel therapeutic targets and biomarkers that can be used to treat such conditions and to achieve tissue regeneration.

We will also study molecules that sometimes hamper healing to the end of blocking them and therefore restore the natural healing process.

Finally, we will study the mechanisms of pain during joint damage and arthritis, so that our interventions also result in rapid pain relief and to prevent the establishment of chronic pain, which tends to persist even if damage is healed.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 need.

Arthritis is the most common cause of chronic disability. Patients with arthritis have joint pain and loss of mobility and lose independence: they often struggle to do their shopping, need help cooking, getting dressed or get in or out of a bath. Patients with arthritis often lose their job because of their condition. Osteoarthritis affects 12% of individuals – up to 40% of all people above the age of 50, is the leading cause of chronic disability worldwide, costing 1.5-2% of the GDP. In spite of being so frequent and disabling, we still have no drug that can stop or revert osteoarthritis and management is limited to pain killers, weight loss – rarely attained – and physiotherapy, until a joint replacement is necessary. Joint replacement restores some degree of autonomy but not a return to an active lifestyle and pain often persists. Therefore, we are in desperate need of novel therapeutics that encourage cartilage regeneration so that we can revert tissue damage in osteoarthritis and return patients to independence, working capacity and an active life-style.

For Inflammatory arthritis, in which inflammation drives tissue destruction, novel drugs are available to stop inflammation, but for those patients in which cartilage or bone damage has already occurred (and this is unfortunately the majority), there is no therapy to encourage healing of these tissues and, even if inflammation is controlled, patients are still disabled and tissue damage progresses due to altered distribution of forces within the joint. Also in this case, therefore, therapeutics that trigger tissue regeneration are essential.

The disabling symptoms of arthritis are joint pain, anatomical damage to joint tissues, and muscle weakness.

Areas of investigation and aims.

Pain.

Pain is the main symptom of arthritis. Pain can be nociceptive (driven by tissue damage) or nociplastic (driven by neuronal plasticity that “rewires” the pain pathways and make patients feel pain even in the absence of tissue damage). Nociplastic pain is present in about half of the patients with chronic arthritis, is difficult to treat and is independent of the degree of activity of arthritis, meaning that even when arthritis is under control, patients will keep having this type of pain.

Therefore, one aim of this study is to study how the molecular events driving arthritis activate pain pathways and how arthritis results in nociceptive and nociplastic pain and therefore how we can treat pain in arthritis.

In the last few years, we and others have discovered molecules which have a function in arthritis mechanisms but also in pain perception. The reverse is also true, for instance, nerve growth factor has an anabolic effect on chondrocytes by inducing the production of Transforming Growth Factor (TGF) beta and Growth and differentiation factor 5 (GDF5).

In the context of this application, the areas that will be covered are:
Identification of cell and molecular mechanisms of nociceptive and nociplastic pain in arthritis.

Identification of modulators that affect regeneration and pain responses.

Tissue damage.

Treating pain is not sufficient in arthritis. For instance, blocking a key pain molecule called Nerve Growth Factor (NGF) in patients with osteoarthritis reduced pain, but, in several patients, it drove a very rapidly progressive osteoarthritis that led to the severe destruction of the affected joints . On the other hand, tissue repair would hardly be beneficial if it wasn't accompanied by pain relief.

Therefore, a second aim of this project is the identification of key molecules that either drive tissue destruction or that promote tissue regeneration, particularly if they also interfere with pain pathways. This is achieved by studying the molecules that govern cartilage and bone formation in development and in painful diseases.

During the tenure of my previous PPL, the same strategy led to the identification of three novel therapies for osteoarthritis which, at least in animals, result in both cartilage regeneration in osteoarthritis and rapid pain relief and the data were published in top scientific journals and attracted vast media attention. We are currently perfecting such technologies to make them suitable for human use and to fully understand their mechanism of action – an essential step for regulatory approval.

In this journey, experiments in rats will be needed to confirm activity in a species with thicker cartilage compared to the mouse and for pharmacokinetics studies.
Several other molecules that have emerged from in vitro or genetics screenings will be tested, mostly in mouse models.

Weakness.

Patients with arthritis develop muscle loss, which contributes significantly to their disability – ability to walk, stand for a long time, open jars, do buttons, operate utensils etc. In addition, muscle loss is a frequent occurrence in old age, even without arthritis. Therefore, a third aim of this study is to identify the molecular mechanisms of muscle loss in arthritis and, ideally, ways to treat it.

The problem of drug delivery.
Cartilage has no blood vessels and therefore it is difficult to reach with drugs. There is therefore a need to optimise drugs and delivery systems so that they reach their target cells and are retained in place.

Therefore, another aim of this study is to optimize delivery and retention systems.

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

Understanding mechanism of tissue formation/regeneration and destruction. Over the years, our studies have revealed mechanisms through which musculoskeletal tissues react to injury and trigger healing responses. Besides the scientific importance, these studies have revealed potential therapeutic targets and have inspired industry in developing therapeutics that exploit the mechanisms of disease that we discovered. In our future studies we will extend this knowledge and thereby provide novel opportunities for the discovery of new drugs and understanding of their mechanism of action, identification of potential toxicity and adverse events.

Identification of novel therapeutic targets. Our past studies have led us to the identification of promising therapeutic molecules that induce cartilage regeneration.

These targets now need to be optimised for human use and several other molecules identified with in vitro studies are awaiting to be validated and studied in vivo.
Validation and improvement of existing molecules to enable their clinical use. For instance, one molecule that we generated lasts for a very short time in the body, which is a problem because it would require daily intra-articular injections. Therefore, adding a stabilizing modification and validate efficacy, mechanism of action and the way the modified molecule behaves within the body is essential. Even when no biochemical modifications are necessary, efficacy studies, dose-finding experiments and characterization of mechanism of action and the dynamics of distribution of the drug in the body and its elimination are required in order to complete the preclinical package and consign such molecules to clinical testing.

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

The academic community. Our studies reveal molecular and cellular mechanisms of tissue formation, healing, and organ development. Therefore, scholars in the field of developmental biology, regenerative medicine, physiology and pathology will benefit from our mechanistic findings.

Patients with arthritis. In the past 15 years we have delivered to the scientific community several therapeutic targets for cartilage regeneration, some of which are being pursued on the long path towards clinical testing. If any of these molecules succeeds, it will be the first molecule to afford disease modification in such a common and so disabling disease as osteoarthritis.

The pharmaceutical industry and the UK economy.

Currently, we have three molecules in the translational pipeline. Even if only one of them was successful, it would be the first-in-kind in the osteoarthritis market which is estimated to be worth USD 11 Billion (MARKETSANDMARKETS).
Society at large. The economy at large: cartilage defects and osteoarthritis cost ~1.5 and 2% of annual gross national product. In 1999/2000, 36 million working days were lost because of osteoarthritis, costing the economy nearly £3.2 billion in lost production (NICE data). Arthritis is the leading cause of absenteeism and disability allowance in the UK.

Companion animals and the veterinary industry. Osteoarthritis is the most frequent joint disease in cats, dogs and horses and is disabling. The dog market for osteoarthritis alone is valued around $4.5 Billion, with 10% of the canine UK population being affected.

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

Publication of detailed methods. We have an excellent track record for publishing our in vivo methodology in great detail so that any researcher around the world can reproduce it. This is done either as extended methodological appendices to our papersor as methodological papers to enable others to replicate and extend our findings.

Dissemination within the musculoskeletal community. Besides actively participating to conference with talks often centred on our in vivo studies, we are part of Centre of Excellence for Osteoarthritis Pathogenesis, which meets for two hours every week to

discuss results and novel findings. Within this forum we advise our colleagues and train those who wish to use our methods.

Species and numbers of animals expected to be used

- Mice: 19100
- 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.

Species.

The mouse is by far our most used species for a number of reasons:
Mice can be easily genetically manipulated. Most of our experiments consist in either increasing or decreasing the levels of a certain molecule/gene. This can be easily done in mice by over-activating a gene or removing it totally or in part. In addition, current technologies enable to delete or activating genes in individual tissues and only when a certain activating drug is administered. This greatly reduces suffering for the animals because, if for instance we are interested in the function of such gene in cartilage, and during osteoarthritis, we can activate the mutation in cartilage only and only after the animal has become osteoarthritic, thereby avoiding all the often very severe effects of the genetic modifications during embryonic development and in other tissues.

Availability of well-validated model systems. Our animal models are highly optimised, with outcomes that are predictable and with narrow variability. This enables us to use much fewer experimental animals to achieve the desired statistical power (typically 0.8) and with minimal suffering.

Availability of reagents and molecular tools. The mouse genome is completely sequenced and well-characterized. Many molecular tools including antibodies and already made transgenic models are readily available, without the need to make them anew and thereby reducing the need of additional experimental animals.

Predictivity. Mouse models have been extensively shown to have a good predictive power for efficacy of therapeutic interventions in clinical trials.

The rat is less convenient than the mouse because genetic manipulation is much more difficult and molecular tools are not as readily available, however, in some instances further validation is necessary (and required by regulatory authorities such as the FDA and MHRA) for the following reasons:

Validation in more than one species and with more than one model greatly enhances predictivity of outcomes in humans.

The structure of the joint surface (and the cartilage in particular) of the rat is more similar to the human one compared to the mouse where the entire articular cartilage is composed of only 5-6 layers of cells.

Because of its larger size, the rat species enables much more accurate intra-articular dosing and dissection of specific tissues for characterization.

Age.

Most experiments will be performed in young-adult animals (i.e. 10-20 week-old mice) because at that stage the logarithmic growth is over, the articular cartilage is mature with a developed calcified layer and mature osteochondral junction. Some studies, however, will be performed, or repeated, with old mice (12-18 months) because homeostatic processes/healing capacity change as a function of age in such models and osteoarthritis is a disease of old age. In addition, aged mice (particularly in the C57BL/6 strain) develop spontaneous osteoarthritis, which is particularly relevant to the human disease which is more frequent in the elderly. Spontaneous osteoarthritis, however, is a confirmatory model rather than a first-choice model due to the higher variability compared to instability-induced models and consequently the need of more animals to achieve the same statistical power. Finally, in some cases, we will want to look at the function of a certain gene in the formation of the skeleton. In this case, pregnant dams will be killed at specific stages of the pregnancy and the embryos will be used for analysis of the skeleton. This is sometimes necessary because many of the molecules that drive cartilage and bone formation during embryonic development also induce cartilage and bone healing in adulthood.
Typically, what will be done to an animal used in your project?

This depends on the question being addressed and therefore the model being used. Breeding and maintenance of genetically altered animals. In most cases, such transgenes are not harmful in standard conditions. The animals will be used for breeding and establishment and maintenance of colonies. Transgenes and knockouts that are harmful, whenever possible, will be in conditional and inducible systems to avoid systemic effects and developmental phenotypes. In such cases, the animals will be normal until they will be put into specific experimental procedures (for instance joint damage and repair) and the transgene will be activated.

Models of inflammatory arthritis. In antigen induced arthritis (AIA), an antigen is injected into a joint and will cause arthritis (pain and swelling) in that same joint which is self-limiting. Bioactive substances or activation of transgenes will be given either before or typically during the progression of arthritis and pain and swelling assessed throughout. The lameness is limited and the arthritis is transient. Therefore, this procedure is classed as moderate. The drawback of this protocol is that there is no tissue destruction taking place and therefore it is suitable to studies effects of therapeutic molecules on inflammation but not on tissue destruction or regeneration. By contrast, in K/BxN serum induced arthritis, serum from X/BxN mice is injected intraperitoneally and will trigger arthritis that affects many joints, with swelling, and pain with small erosion of cartilage and bone, as in human rheumatoid arthritis. This protocol is classed as moderate and the arthritis is transient, but is slightly more severe than AIA. Therefore it will be performed, only when AIA is not possible: when we need to assess tissue destruction.

Osteoarthritis models and models of acute cartilage injury. This procedure aims at studying the effects of a certain molecule/gene in the context of osteoarthritis or to study mechanisms of homeostasis in osteoarthritis or cartilage degeneration. These are the most commonly used procedures. The animals undergo a surgical intervention under general anaesthesia (most commonly resection of the anterior part of the medial meniscus and of the medial collateral ligament to induce osteoarthritis or generation of an acute osteochondral defect). For about 3 days after the operation the mice suffer some pain

which can be detected by the fact that they load about 5-10% less body weight compared to the contralateral limb, but there is no evident gait change or lameness. They rear spontaneously and climb the cage as normal. After 3-4 days they have no measurable pain anymore until about 7 weeks after the intervention when, again, they start loading less body weight on the operated limb (about 5% less than the contralateral limb). The animals are then killed typically 10-16 weeks after surgery. An intervention (injection of a therapeutic molecule, or injection of tamoxifen in the case of inducible transgenics) is typically given either before (preventive regime) or after (therapeutic regime) surgery. Peri-operative Analgesia will be given whenever possible, however, sometimes it will be limited to skin anesthesia because: 1. Pain is often an important outcome of the experiment; 2. essentially all the known molecules that targets of pain killers also alter the behaviour of cartilage cells; 3. as shown by our measurements by sophisticated instrumentation, the amount of pain in these models is minor and is not associated with any lameness or altered behaviour. Pain can be measured at regular intervals using non-invasive, well-tolerated procedures. To reduce to a minimum animal stress, the animals are regularly handled before the procedure so that they get accustomed to the investigator and the equipment. This also reduces variability and consequently increases statistical power.

Cartilage formation assay in immunodeficient mice. In this procedure, cartilage cells or stem cells from another species (typically human) are injected either intramuscularly in the thigh or implanted subcutaneously on the back or in the ear pinna, of immunodeficient mice. In the case of the intramuscular injection this is done without anaesthesia, whereas the subcutaneous implantation requires anaesthesia. After implantation the mice may receive a local (where the chondrocytes have been implanted) or systemic administration of a therapeutic substance. The chondrocytes will form small cartilage organoids that are usually retrieved 2-8 weeks after implantation, and which are painless. Besides the anaesthesia (when needed) and the injection, this procedure is not stressful to the animals.

Ear pinna healing. This procedure is used to assess whether a molecule affects cartilage healing or cartilage biology without having to induce joint damage. It can also be used to assess whether a molecule engages with chondrocytes. It is used when we want to isolate the effects on homeostasis from those linked to inflammation and catabolism. Typically, a regular ear punching hole is generated the same way it is normally done to ear-mark mice, and a substance is injected within the dermis of the ear pinna, between the skin and the cartilage to see if it promotes healing. In some cases, it is not necessary to perform the ear punch when the experimental question resides in local effects on the underlying cartilage (e.g. proliferation, differentiation etc.). The procedure is done under general anaesthesia, unless the therapeutic molecule is delivered as a topical application or systemically, rather than a local injection.

Muscle regeneration. In this protocol, a toxin, typically cardiotoxin, is injected within a muscle of the leg and, for about one week, will cause muscle pain. Subsequently a bioactive substance (for instance a molecule that we think is going to facilitate repair) is injected either locally or systemically. Since the tibialis anterior is only one of the muscle that dorsally flexes the ankle, however, this does not result in “foot drop” and the animals have unrestricted mobility.

Assessment of phenotypes in inducible transgenics. This protocol aims at observing the effect of a gene modification on either the way the skeleton develops or on the development of spontaneous osteoarthritis. In this protocol mice pregnant with genetically modified pups may be injected with tamoxifen to activate the gene modification and killed at different times of pregnancy. The pups will be killed immediately and processed to

identify skeletal defects. When there are no effects of gene modification detected during development, or if these effects are very mild, the pups may be allowed to be born naturally and may be allowed to develop and age up to the age of 18 months. At the end of the study all animals will be killed, and the joints will be processed for analysis of the skeleton.

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

Joint surface damage and repair. In this protocol, a joint surface injury is made by either directly generating a surgically defect in the cartilage and bone defect, or indirectly by destabilizing the joint (typically resecting the anterior part of the medial meniscus and the medial collateral ligament). During the first week the animals will have some pain on weight bearing which can be measured using incapacitance or von Frey filaments, but not such to cause lameness or alteration of gait visible by eye. Typically, the animals are then pain-free until six weeks later when pain is measurable again with specific and sensitive tools but does not result in lameness or any visible alterations of gait.

Models of inflammatory arthritis.

In antigen induced arthritis the animals suffer from pain and swelling to the injected joint only, which results in some lameness but is of moderate entity and does not limit the capacity of the animal to get to their food.

In K/BxN induce arthritis the side effects are similar to that of antigen induced arthritis, however the arthritis will involve several joints at the same time. The arthritis is likely to be more severe than in antigen induced arthritis but will not limit capacity of the mice to get to their food.

Ear pinna healing. In its typical form, the animals in this protocol will be subjected to a defect to the ear pinna as per regular ear-marking and molecules will be tested for their capacity to improve healing. Such molecules may be given systemically, topically over the skin, or by a local injection between the skin of the ear and the cartilage. Anesthesia will be necessary if the molecule being tested is applied or injected locally to the ear. Apart from the pain of the ear-marking procedure, therefore, there might be some local inflammation. In some cases there will be no need to make a defect in the ear pinna: for instance if we are simply interested in the effect of a molecule on the cartilage.

In all protocols requiring anesthesia, there is a chance that some mice may suffer from cardiovascular accidents. This is exceptionally rare with young mice, but may be more frequent with older mice and mice fed with high fat diet.

Tissue formation assay in nude mice. In this protocol, cells are injected - or implanted as a pellet - under the skin or within the muscle of immunodeficient mice.

Substances can be delivered to test their ability to promote tissue (typically cartilage) formation starting from the implanted cells. Apart from the procedure of injection (no anesthesia required) or implantation under anaesthesia, no pain or discomfort is expected. There is a very small risk of infection to the site of implantation, but the applicant, who has performed this model ~ 1000 times over the last 20 years, has never seen it happening, except for the occasional evidence of pockets of inflammatory cells within the implants after dissection.

Muscle regeneration protocol. In this protocol mice will be injected into the tibialis anterior muscle with cardiotoxin. The mice will have muscle pain for a few days which will not compromise mobility or access to food.

Effects of knee surgery.

The most common side effect is that the mice undo the skin suture. To prevent this from happening, the suture is performed in such a way that the knot is buried underneath the skin and therefore is less accessible. One day after surgery all mice are checked and, whenever necessary, re- sutured. They are checked again the following day and if they have again managed to open the suture the wound is left to heal of second intention under the supervision of the NVS.

As with any surgical procedure, there is a theoretical risk of infection. However, the applicant has performed this procedure several hundreds of times and has never observed septic arthritis.

Tamoxifen injection. In all protocols in which conditional KO or transgenics are used, additional side effects can come from the intraperitoneal injection of tamoxifen to activate inducible transgenics. Usually, in young mice, tamoxifen injection is associated with malaise and mice being “slow” for a day or two. In old mice, however, tamoxifen needs to be carefully titrated because it can lead to severe side effects including mortality, usually for cardiovascular toxicity.

Breeding and maintenance of genetically modified animals with a harmful phenotype. Here the harm is dependent on the phenotype of the genetic modification. As we are interested primarily in cartilage and bone, typically these mice, when the transgene is activated during development, may have altered gait and somewhat deformed limbs. In most cases, however, the mice will just have a predisposition to develop osteoarthritis, with some pain to their joint.

Expected severity categories and the 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	Severity	Proportion
Breeding and maintenance of GA animals	mild	100%
Breeding and maintenance of GA animals with harmful phenotype	moderate	100%
Inflammatory arthritis with modest tissue damage	moderate	100%
Joint surface damage and repair	moderate	100%
Tissue formation assay	moderate	100%
Ear pinna healing	moderate	100%
Muscle regeneration	moderate	100%

Assessment of phenotypes in genetically modified animals	moderate	-50%

- Killed
- Used in other projects

Replacement

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

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

We have developed and optimized many in vitro systems to replace a vast number of animal experiments in the following contexts.

In vitro cartilage or bone formation assays to screen for chondrogenic molecules.
Wound healing assays to measure the capacity of cells to fill a gap in culture or to migrate in response to biochemical stimuli.

Cartilage degradation assays. These assays measure the capacity of molecules to antagonize the catabolic effects of inflammatory molecules or oxidative stress.
All these models allow us to replace many animal experiments by screening out molecules that do not interfere with cartilage formation, wound healing, inflammation and tissue damage, and to understand how these molecules work. Animal experimentation is necessary to identify the truly reparative molecules and to identify potential adverse events that do not take place in vitro: for instance, bone morphogenetic proteins (BMPs), in a test tube cause a lot of cartilage formation and therefore one may think that they would be good in osteoarthritis, however, when injected in a real joint of a live animal, they cause formation of cartilage and bone all over the place, not respecting the anatomy of the joint and therefore leading to deformity and loss of mobility.

In spite of their utility in screening and in understanding molecular mechanisms, such assays alone are insufficient and in vivo confirmation is almost always required: in some cases, for instance, in vitro results may even be misleading: for instance transforming growth factor beta (TGF-β) is a very potent inducer of cartilage formation in test tubes but when administered in animal or following forced expression it actually results in osteoarthritis and cartilage destruction and its blockade improved osteoarthritis outcomes.

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

In vitro cartilage or bone formation assays to screen for chondrogenic molecules: this model involves culture of stem cells or chondrocytes in three-dimensional micromasses. Chondrogenic molecules such TGF beta or BMP-2 will induce these stem cells to turn into cartilage cells and activate cartilage-specific genes.
Wound healing assays to measure the capacity of cells to fill a gap in culture or to migrate in response to biochemical stimuli. In this assay, mesenchymal cells are cultured in vitro and a controlled gap is created. The speed at which the cells fill the gap is measured and compared following treatments.

Cartilage degradation assays. These assays measure the capacity of molecules to

antagonize the destructive effects of inflammatory molecules. For instance chondrocytes can be cultured and exposed to inflammatory molecules such as TNF alpha or TGF beta and the proteoglycan content or release be measured.

Why were they not suitable?

As mentioned above, these assays are very useful, and we always use them for screening and in order to study molecular mechanisms, but they only look at a subset of the factors that contribute to chondrogenesis and repair; very often therefore, in spite of brilliant results in vitro, once tested in vivo, results can be unpredictable and even the opposite of what we expected from in vitro experiments.

For instance:

Suppression of calmodulin kinase II (CaMKII) seemed a great therapeutic option for osteoarthritis based on in vitro assays, but in vivo, mice deficient in CaMKII were predisposed to osteoarthritis through a mechanism involving induction of catabolic enzymes.

WNT16 activated WNT signaling in vitro but actually inhibited it in vivo, due to competition with more potent WNT molecules and therefore, unexpectedly, its deletion made mice more susceptible to osteoarthritis.

TGF-beta is a very potent inducer of chondrocyte differentiation and cartilage formation in vitro but when administered in vivo or following forced expression it actually resulted in osteoarthritis and cartilage destruction and its blockade improved osteoarthritis outcomes.

No in vitro assay is able to model the effect of cartilage damage on pain.

Reduction

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

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

Maintaining genetically modified animals contributes to the majority of the experimental animals. Large colonies are required to obtain sufficient number of animals of the desired genotype at the same time. In addition, we are currently working on 6 molecules and additional molecules will join our portfolio within one year. These molecules will be studied in osteoarthritis, cartilage repair models, inflammatory arthritis models and its variants. Depending on the model, the number of animals per group (and per gender) varies from 10 to 22 animals in order to reach 0.8 power, plus contingencies (for instance, fighting within the cages, infections etc.). Each of these will require multiple dosing and different time points. Maintaining genetically modified animals contributes to the majority of the experimental animals also taking in consideration the inevitable genotype discharge.

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

For each experiment a clear primary endpoint and secondary endpoints are identified. This enables us to obtain parameters (relevant difference of means, variance, expected outliers and failure rate) that are essential to perform a proper power calculation. In this regard, we pre-define which of the outcomes are essential and the number of animals per group is calculated so that all such essential outcomes will have a power of at least 0.8.

Prioritization of the in vivo models. Mild and more consistent protocols (e.g. ear punch healing in genetic screens) will reduce the number of more invasive procedures. Equally, when possible, adenoviral overexpression may be used before embarking in extensive crossing to generate conditional or inducible transgenics.

Availability of highly optimized and specialized models. We have a wide range of optimized animal models. This allows us to select the model that best fits the circumstance, allowing us to use more severe models for resistant strains and vice versa. This allows to achieve a higher statistical power and therefore reduce the number of experimental animals, but also to avoid using models that are too severe in strains that are sensitive.

Careful planning of the experiments. Our expertise with animal models allows us to perform accurate power calculations to determine the minimum number of animals necessary to achieve a statistical power of 80% in accordance with ARRIVE guidelines using our own previous data to derive variability and expected effect size.

Use of multiple validated readouts at repeated time points. This approach increases the statistical power. For instance, the use of histomorphometry is more powerful than a semi-quantitative osteoarthritis score. Also, the use of longitudinal measurements at multiple time points (e.g. pain measurement, measuring luminescence or fluorescence, imaging in vivo in reporter mice) avoids in many cases the need to set up multiple groups of mice to be killed at different time points.

Use of internal controls whenever possible. Internal controls (for instance untreated contralateral leg) allow to improve the power of statistics by eliminating the variability due to the individual animal. Equally, performing ear punch on the mice used in protocol “ear pinna healing” will not only reduce the number of experimental animals, but also allow to discriminate between a genetic trait and an environmental effect.

Use of inbred mice. Using inbred strains allow for much smaller variability and therefore to achieve a higher statistical power and smaller experimental groups.

Within our group and collaborators we have sufficient statistical expertise. However, we constantly seek advice from other colleagues and statisticians to improve experimental design.

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

Breeding schemes are carefully planned so to keep colonies as small as possible, whilst allowing efficient experimentation and to maximise the number of pups with the desired genotype with the least number of crosses.

We perform pilot studies and single dose escalation studies with surrogate response markers to obtain data necessary for power calculation and restrict the doses and frequency of administrations.

The issue of fighting within cages: fighting within the cages are often cause for increase severity of osteoarthritis and worse outcome, which results in increased variability and therefore lower statistical power and potential introduction of bias. This is often obvious when plotting the outcomes by cages, regardless of treatment. To avoid this problem we take the following measures: a) We frequently monitor cages for signs of fighting. In the case of fighting, we try to identify the “culprit” – usually the mouse with the coat in best conditions – and we remove it from the cage; b) We randomly attribute an equal number mice within each cage to receive treatment or control (block-randomization), so that, if fighting takes place, it affects all treatments and so that we can take this into account when we perform a statistical analysis. This enables to control for such potential bias and therefore contain the number of animals per group needed to achieve power > 0.8.

Data sharing. We routinely replace several animal experiments by re-analysing raw data available online in "big data" repositories such as the Gene Expression Omnibus (GEO - https://www.ncbi.nlm.nih.gov/geo/). Reciprocally, we deposit on GEO or other appropriate repositories any big data suitable for sharing. Finally, we publish routinely as supplementary materials all the raw data and statistical analysis scripts for our experiments. This enables other researchers to mine our data for their experiments and also to use our raw data to best power their own experiments.

Refinement

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

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

Models of osteoarthritis, rheumatoid arthritis or joint surface repair are highly strain sensitive. Therefore, it is important to be able to use models that have the appropriate level of severity for each strain. This will also avoid unnecessary suffering (e.g. by using a particularly sensitive strain on a relatively more severe model) or having to increase the number of experimental animals in order to reach adequate statistical power. For instance, DBA1 mice are resistant to instability- induced osteoarthritis while 129SV are very sensitive. Therefore, if we need to test the capacity of a molecule that induces cartilage formation in osteoarthritis and we have a transgenic mouse in the DBA/1 background, we will have to use a more severe model (MLI instead of DMM). Vice-versa, if we want to see if a certain KO in the 129Sv background develops more severe osteoarthritis we would rather use the DMM.

Use of mild protocols when screening is required. For instance, when appropriate, using the ear punch healing assay for genetic association before the more severe protocol for joint surface damage and repair. This choice will be made on a case-by-case basis.

Use of the mildest protocol that allows to obtain the information sought with the highest statistical power. For instance, to achieve instability-induced cartilage breakdown in sensitive strains (e.g. 129Sv or C57Bl/6) the destabilization of the medial meniscus may be

preferred to the resection the medial collateral ligament + removal of part of the meniscus. In resistant strains such as FVB or DBA/1 the latter will be preferred, not only because it will be more suitable to measure cartilage breakdown, but also loss of statistical power due to the fact that most animals would be left with hardly any cartilage at all to start with (ceiling effect), which would require more experimental animals.

Careful experimental design: for instance the use, when possible, of internal controls, appropriate time points that maximise outcome and reduce variability, and, whenever possible use of longitudinal outcomes (multiple measurements in the same animals) that allow avoiding setting up separate experimental groups at different time points.
When possible and scientifically appropriate, we will use tissue-specific and inducible knockout or overexpression systems: these are systems in which the genetic modification only takes place in the tissue of interest and only at the time of the experiment. These systems therefore enable to eliminate the suffering due to effects on other tissues and to congenital malformations.

Maintaining and developing surgical skills and expertise in animal husbandry. The applicant, has more than 25 years expertise with sophisticated surgical models in mice. In our group a lot of emphasis is placed in ensuring that this expertise in maintained and passed over to junior researchers, together with the understanding of the principles of animal handling and husbandry. This effort not only ensures high quality results, but, in my opinion, it is the most important factor in reducing to a minimum animal suffering.

Unfortunately, often we will not be able to make use of analgesics in our models because pain is not only an outcome that we need to measure (clearly, if we are assessing whether a certain molecule causes pain relief, we cannot give pain killers at the same time), but also affects cartilage destruction: For instance, NGF, which mediates pain, is also a cartilage growth factor. Indeed, the potent NGF inhibitor and pain suppressor Tanezumab in osteoarthritis was suspended because, in spite of the extraordinary efficacy in suppressing pain, it accelerated osteoarthritis progression in terms of cartilage damage in a subset of patients.

http://www.fda.gov/downloads/AdvisoryCommittees/CommitteesMeetingMaterials/Drugs/ArthritisAdviso ryCommittee/UCM295205.pdf

Finally, chondrocytes express functional opioid receptors and respond to opioids by activating inflammatory pathways.
Therefore the use of analgesics may introduce severe artefacts. In addition, pain, at least in the non- inflammatory models, is generally mild and even immediately after recovering from the anaesthesia, mice walk normally and rear as normal.

In “inflammatory polyarthritis”, animals can have pain to multiple joints including the temporomandibular joint with consequent problems in eating. We will ensure that we take measures to reduce suffering by providing:

Soft litter, to reduce pain on walking.

Sufficient soft, non-tangling nesting material to keep comfortable, cushion sore joints and enable thermoregulation.

An appropriate group of cage-mates for social animals, depending on age, sex and strain.

One or more refuges, to permit natural behaviour and alleviate potential anxiety in animals

with compromised mobility.

Effortless access to easy-to-eat food and water, to cater for disability.
Soft food, to counteract or prevent weight loss and to help with mastication for those mice who develop arthritis to the temporomandibular joint.

We will take proactive welfare management as opposed to reactive. For example, animals will be acclimatized to cage provisions, appetizing food and hydration agents before arthritis is induced. We will be very gentle with handling and avoid pulling their tails. When appropriate, hand cupping and/or tube handling will be used instead.

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

Our focus is the damage and regeneration of joint tissues with a focus on the knee. Clearly, we cannot use invertebrates as they have no skeleton. Zebrafish do not have synovial joints apart from the temporomandibular joint, which is not amenable to models of osteoarthritis, and which does not share much with the structure of mammalian joints: the entire condyle of the temporomandibular joint of a zebrafish is composed of just one cell. Therefore, the mouse is the least sentient animal in which knee osteoarthritis models are meaningful. For some studies, the rat will be better owing to the structure of the articular cartilage being more similar to that of humans and to the fact that intra-articular dosing is more accurate due to the larger size.

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

By constantly improving our own models: for instance, the joint surface injury model required >20 animals per group to achieve 80% statistical power and was burdened by up to 40% occurrence of patellar dislocation, whereas our recent improvement requires half the number of mice and has no occurrence of patellar dislocation. We also consign these improvements to the wider community by describing in great detail our improvements in the methods section of our publications, by training other academics as well as investigators from industry in our models and best practice.
By timely implementing such improved protocols.

By ensuring that all personnel is trained to the highest standards and is sensitized to the issues of animal husbandry and handling of the animals. For instance, we train our personnel to minimize as much as possible picking up the mice by the tail and we ensure that the animals are handled frequently, which greatly minimizes their stress. We believe that, in an animal facility, the single greatest variable affecting the welfare of the animals is the quality of the handling and care they receive from the investigators and personnel in charge of them.

By frequently monitoring the animals' health status and liaising frequently with the NVS and the BSU personnel.

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

We routinely follow ARRIVE guidance for reporting and experimental design. To maximize refinement in our experiments, we use the PREPARE recommendations and other relevant literature.

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

Our group is leading in the area of in vitro models of chondrogenesis and we collaborate with other groups that also develop in vitro models of repair/regeneration. Therefore, we manage to perform the vast majority of mechanistic experiments in vitro rather than in vivo.

By keeping up to date with the 3R literature through regular bibliography searches and quick implementation of the advances. For instance, the ear pinna healing model is a recent acquisition for our group and will enable us to reduce the number of animals massively owing to the opportunity to do repeated time points in the same animals rather than sacrificing at different time points multiple groups of animals and also, when possible instead of injury models in the knee, is also much less stressful/painful for the animals.

By checking the NC3R website and reading the updates supplied by our BSU and AWERB personnel.

7. Gene regulation during cell fate specification

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

Gastrulation, Stem Cells, Germ layers, Embryo, Development

Animal types	Life stages
Sheep	adult, pregnant
Pigs	adult, pregnant

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The overall aim of the project is to determine the role of genes responsible for specification of basic germ layers (e.g. mesoderm, endoderm) during pig and sheep embryogenesis.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 determine how cell fate commitment of pluripotent cells is controlled at the onset of embryo development and will evaluate the key roles of specific genes during specification of basic germ layers in vivo.

This new knowledge will help us define the genetic basis of early embryonic loss, as well as some pathological conditions, such as germ cell tumours.
Better knowledge of the genes regulating early embryonic development will help researchers develop new protocols for the in vitro differentiation of embryonic stem cells

(ESC) into cell types of therapeutic interest. These will be useful tools for investigations into chromosomal abnormalities, and the effect of endocrine disruptors and environmental contaminants on fetal development. This new understanding will contribute for example: 1- to creating more suitable models of human disease, 2- to perform toxicological screening and 3- to assess how cells can become cancerous upon exposure to different chemicals.

Another outcome of the project will be the generation of germline competent livestock ESC (sheep and pig), which will be useful tools for the generation of genetically modified animals. These will be valuable research resources in the short to medium-term for disease modelling, and longer term for use as a source of organs for Xenotransplantation and for drug screening. Pig ESC will be used by researchers modelling and biotech companies with interest in modelling human diseases.

Good knowledge of how ESC can be established will also help with a better understanding of the conserved pluripotency pathways in mammalian embryos.

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

The results of the work will be published in academic peer-reviewed journals (scientific publications).

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

The research plans described in this license are timely and important because they will study how the basic embryonic tissues are formed during pig development. The pig is a relevant species that shares similarities with human development and therefore information from pig embryos can inform on human developmental biology.

The knowledge of how specific tissue progenitor cells form in mammals comes from studies in mice, however recent studies using human embryonic stem cells demonstrated that mice and human have very different developmental programs. However, investigations using human embryonic stem cells are not sufficient to demonstrate what happens in a human embryo, and in vivo validation of in vitro findings is a scientific imperative. In vivo validation cannot be done with human embryos, therefore studies in pig embryos can be used to infer shared developmental mechanisms in species with similar embryology. The fundamental new research proposed under this license aims at establishing a new paradigm in our knowledge of how gastrulation ensues in humans and other mammals with conserved embryology. This research is timely, because recent publications demonstrate the close developmental similarities between pigs and humans. The new knowledge on how specific gene programs regulate development will lead to a re-write of developmental biology textbooks, and will have wide implications of our understanding of developmental abnormalities affecting pregancy establishment during the first weeks after conception, as well as better knowledge of the causes of infertility. A better understanding of how germ cells develop in humans is critical for establishing how environmental factors can affect health status of a baby. For instance, the incidence of germ cell tumours is increasing in the Western world, but it is not clear what are the causes and the underlying mechanisms affecting germ cell development. Identifying the vulnerable periods of germ cell development and how they may be affected by, for example, exposure to environmental pollutants will give clinicians new avenues for investigating these disorders.

As part of the program of work this project will evaluate the potency of pig and sheep embryonic stem cell for their differentiation in a fetus. This new technology will benefit

many researchers using pigs and sheep as model system for the study of human diseases, for which it is necessary to generate a transgenic animals. At present this can be achieved via animal cloning, however the efficiency is low.

Using farm animal ESC will accelerate and increase the efficiency of producing founder transgenic animals.

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

All the results will be published in peer reviewed journal, and via pre-print servers, to accelerate the dissemination of the work. Collaboration with colleagues from other institutions will be promoted through presentations at scientific conferences too. Generation of mutant embryos may yield phenotypes that may be relevant to investigations in areas of developmental biology in which we may need expertise from collaborators. We will seek to work with experts in these areas to further investigate the mutant phenotypes.

Species and numbers of animals expected to be used

- Sheep: 60
- Pigs: 66

Predicted harms

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

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

We have chosen the pig as a model species because of the developmental similarities with human. To determine the role of key genes involved in early lineage specification it is essential to use embryos from species with relevance to human development, so that we can understand the basis of early embryonic loss after conception. Furthermore, the pig genome is fully sequenced, and allows us to perform precise genetic modification. The pig is also a species of important biotechnological importance. Findings from our work may also contribute to increasing the biotechnological applications of pigs for biomedical research and animal production. We will use female pigs to perform embryo transfer of gene edited 1-cell zygotes.

We have also generated livestock (pig, sheep and cattle) ESC in our laboratory which will be used to generate genetically engineered animals in future. It is therefore necessary to test the pluripotential capacity of these cells in the host embryos. To do this work we will use sheep embryos. The advantage of sheep as a species is that IVF work very well, in contrast to pigs. In vitro generated Morulae/Blastocysts will be injected with sheep stem cells and transferred into recipient ewes to assess chimeric contribution. The validation of pig stem cells in chimeric embryos will be carried out after collecting day 5 moruale from female pigs on day 5 after artificial insemination.

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

Gilts and ewes will be oestrus synchronized prior to embryo transfer of day 1 zygotes (pigs) or day 7 blastocysts (ewes). Embryo transfers will be done by laparotomy. To do this

the animal will be sedated and intubated to induce deep anaesthesia. After the operation (~30-45 minutes) the animals will be transferred to a recovery room where they animals are expected to make a rapid and unremarkable recovery from anaesthetic.

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

No major adverse effects are associated with oestrus synchronization of gilts and ewes. A mild discomfort may be cause by the introduction of the intravaginal sponge. It is important to lubricate the applicator to avoid rubbing. No more that mild temporary discomfort may be caused by artificial insemination procedure in pigs.

Surgical procedures will be carried out aseptically. In the uncommon event of post-operative complications, animals will be killed unless, in the opinion of the NVS, such complications can be remedied promptly and successfully using no more than minor interventions. In the case of wound dehiscence, uninfected wounds may be reclosed on one occasion within 48hrs of the initial surgery. Peri and post-operative analgesia will be provided; agents will be administered as agreed in advance with the NVS. Animals are expected to make a rapid and unremarkable recovery from the anaesthetic. Any animal that fails to do so would be killed by a schedule 1 method.

Humane endpoints:

In the unlikely event that animals exhibit signs of pain that cannot be controlled by the use or analgesia, or develop infection that does not respond to antibiotic treatment, they will be killed by a Schedule 1 method.

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

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

Both for pig and sheep embryo transfers by midline laparotomy is categorized as moderate severity.

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

- Killed

Replacement

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

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

The study is based on using pigs because it is the most suitable animal for modelling early human development. The program of germ layer formation during gastrulation is different in mice, therefore it cannot be used as model for humans. Non-protected animals (invertebrates) have very different modes of germ layer development, and are not relevant to humans.

The period of embryo development (Day 12 and Day 35) of the study cannot be replicated in vitro, therefore it is essential to perform embryo transfers. Ewes are used in this study because IVF in this species is highly effective. These embryos will be used as recipients of stem cells that will be assessed for their capacity to contribute to a fetus. To do this we need to transfer embryos into a sheep and carry the pregnancies until the fetus is fully developed.

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

We have recently started using gastruloids, which mimic processes of embryo development as a mean to screen for phenotypic features of mutant stem cells. We aim to develop this further to use it as a platform to describe certain properties of gastrulation. However, this system is in its infancy and does not completely recapitulate embryo development. Validation of the findings in gastruloids require confirmation in embryos. The use of gastruloids will help reduce the number of embryos required to determine a genetic mutant phenotype.

Why were they not suitable?

As indicated above, the current state-of -the art of gastruloid technology does not allow for a comprehensive characterization of the mutant phenotype. Whilst useful as a first screening option, validation in embryos is essential to establish the correct phenotype of the mutants.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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, this have been estimated on based on the number of genes we want to study and the efficiency of embryo transfer in both species.

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

Embryo transfer are well established methods that yield large number of embryos in pigs, which help minimize the need for many sows.

We use multiple embryo transfer (between 25-40 embryos) to a single recipient after gene editing, and since gene editing of zygotes is a highly efficient procedure (>50% bi-allelic KO) and the survival after manipulation is very high (>80%) we can obtain mutant embryos from a small number of recipients.

The number of surviving embryos is estimated at around ~60%, and the proportion of mutant embryos >50%. Thus, from one recipient animal we expect to obtain between 10-15 gene edited embryos. More than 90% females will maintain pregnancies for 13-14

days. This design will ensure that we obtain a minimum of 10 mutant embryos per female. Three embryos will be analysis to confirm the reproducible phenotype of mutant (KO) embryos, following the principles of reproducibility in experimental design.

Based on the calculations presented above, we need 3 recipients per gene and we will study 6 genes. Thus, the combined total number of animals for this objective is 18 recipients.

Power calculation was used to estimate the animal requirements for the project. For pigs we assume a probability of success of 1% (1 foetus/100 showing chimeric contribution) and Power= 80%. Based on these assumptions we need to generate 185 embryos to determine the ability of a cell line to contribute to a chimera. We will transfer ~20-25 embryos/recipient and we expect >90% pregnancy. We need 10 recipients/cell line and we will test 4 cell lines. A total of 48 recipients will be needed to test 4 cell lines (we will oestrus synchronize an additional 20% to account for those animals not responding to oestrus synchronization).

For sheep we assume a probability of success of 1% (1 foetus/100 showing chimeric contribution) and Power= 80%, however 8-10 IVF embryos will be transferred per ewe. We expect 90% pregnancy rate based on previous results by Sinclair et al., 2016. Thus, we need 12 recipients/cell line, and we will test 4 cell lines. A total of 60 recipients will be needed to test 4 cell lines (we will oestrus synchronize an additional 20% to account for those animals not responding to oestrus synchronization).
By choosing pigs and sheep as experimental species, which can carry multiple embryos, and suitable methodologies (embryo transfer, gene editing, chimeric embryos) we are able to reduce the number of animals used to answer the questions addressed in 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?

The animals used as embryo donors for our research aims will be used by other groups using pigs on our campus. Typically, different tissues and organs are also used for studies on joints (arthritis research) and digestive system (gut content). Thus, we coordinate activites such way that we maximise the beneficiaries of the animals tissues available from our 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.

We have chosen the pig as a species because pig and human embryos have very similar development. To determine the role key genes involved in early lineage specification it is essential to use embryos from species with relevance to human development.
Furthermore, the pig genome is fully sequenced, and allows us to perform precise genetic

modification. The pig is also a species with important biotechnological importance. Findings from our work may also contribute to increase the biotechnological applications of pigs for biomedical research and animal production.

The sheep is also chosen as a model because it shares developmental similarities with human embryos, and has the advantage that IVF is very efficient. Different human diseases to those modelled in the pig can be generated in this species, therefore depending on the scientific rationale experiments will be done in sheep or pig accordingly.

Pig and sheep ESC can be used to generate genetically engineered pigs in future. It is therefore necessary to test the pluripotential capacity of these cells in the host pig/sheep embryos.

For genetic modification (Objective 1) we will use Day 1 zygote injection of gene specific guide RNAs and Cas9 RNA/protein. Injection of 1-cell embryos maximizes the chances of gene editing and reduces the proportion of non-targeted cells, minimizing the number of embryos required to obtain a bi-allelic knock out.

For the generation of chimeric animals we will use Day 5 morula as recipient embryos. Experience from other species demonstrates that morulae are best suited for incorporating cells into the inner cell mass of a blastocyst, therefore increasing the probability of integration of pig and sheep ESC into host embryos.
Procedures to minimize animal suffering:

Surgical ET procedures (Protocol 2): For objective 1 we need to transfer embryos on D1, which requires ET to be performed into the oviduct. For objective 2 we will transfer blastocysts, which according to some experimental reports can be transferred transvaginally. However, this technique is not very efficient and is not recommended for experimental embryos, such as the chimeric embryos generated in this project. Surgical ET by midline laparotomy is the preferred method that ensures a rapid and reliable intervention for both embryonic stages. This will be performed under general anaesthesia in our dedicated operating theatre under aseptic conditions. A small midline laparotomy will be performed to exteriorize the oviduct and perform embryo transfer using a 3.5 French gauge tomcat catheter. The procedure should take around 30 minutes. Animals will be given peri-operative analgesics followed by post-operative antibiotics and analgesics to prevent systemic infection and pain, respectively. During the first 48 hrs animals will be housed individually and monitored twice a day to look for signs of discomfort or pain (e.g. postural changes, gait, apathetic, unwilling to move or absence of bed making behaviour). After this period animals will be housed in small groups for the remaining of the period (~3 weeks).

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

We have to generate mutant embryos that grow for several days or weeks. After embryo transfer the recipients will carry out normal life, and they will be subject to schedule 1 to enable embryo retrieval. There is no other alternative way to create embryos ex-utero at present.

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

From our recent experience in performing this work we have evidence of a very high standard of care of the animals undergoing surgery. All animals recover very well soon

after surgery and are grouped with other animals within 24 hrs. To minimize discomfort animals are given painkillers during this period and they are monitored 4 times a day. Animals are always kept in close contact with other animals, although physically separated to avoid disturbance post-surgery. The animals can see each other and are regularly (3-4 times a day) checked by staff.

Veterinary intervention will be promptly sought as required and appropriate humane endpoints applied in the unlikely event that adverse effects develop and cannot be controlled. Full records of procedures undertaken, daily monitoring and veterinary requests will be maintained.

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

Home Office Code of Practice will be used to ensure animal care and housing is appropriate. This guidance will be used in conjunction with the advice available in the NC3Rs Resource Hub for both housing and handling of animals.

The NC3Rs' Procedures with Care resource will inform personal licensees of refinements in the conduct of the minor procedures undertaken in this licence.
Home Office and FELASA severity information will be used to ensure that the actual severity experienced by the animals can be recorded and limits within this licence adhered to.

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

I read the literature and incorporate suitable methodologies to my research program in order to reduce the usage of animals. An example of this is the adoption of gastruloids to our research pipeline, which has yielded excellent results and is now been used to screen for phenotypes before we decide to use embryos for transfer into recipients. I also keep myself updated on the latest NC3R activities by reading their work on their website and through academic channels.

8. Gut development, disease and regenerative medicine

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

Gut, Motility, Developmental biology, Disease, Regenerative Medicine

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim of this project is to investigate how the gut develops, understand what happens in gut development to cause disease, and to develop better treatments for gut 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 correct functioning of the gut depends on the movement of the gut wall which allows food to move along the gut. This functional movement is called "motility". Motility in the gut is caused by the actions of a number of different cell types including nerve cells, muscles and a cell type called interstitial cells of Cajal, or ICC for short. When there is an issue with any of these cells, this results in diseases called "gut motility disorders". Unfortunately, clear data showing number of people with motility disorders in Europe is not known. However, within the United States, motility disorders led to 106,000 hospital admissions in 2018 with an annual cost of $3.1 billion. Futhermore, there are no cures for these

diseases. Due to this, treatment remains a challenge and approximately 60% patients often have surgery to remove large pieces of gut. This can cause significant problems, as patients must live with long-term, life altering symptoms with 14.2% of patients requiring hospital readmission . Hence, there is a critical need to develop alternative approaches to treat these diseases. This project is important as it will provide information on how the cells of the gut develop. It will also allow us to better understand gut disease and develop new, and better, treatments for people with gut motility disorders.

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

This project will create new information and data on the development of the gut, gut motility disorders and regenerative medicine, including cell therapy approaches, for gut diseases. Project data, and study datasets will also be published in open access journals, and data repositories where appropriate. By the end of this project, we hope to generate various cell therapy products for the treatment of gut motility diseases.

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

The proposed research outcomes will contribute significantly to our understanding of the functional development of the gut, along with developing novel approaches to treat intestinal diseases. These outcomes are likely to benefit researchers (in the UK and internationally) and the wider scientific community with long-term benefits to both patients and the NHS as outlined below:

Researchers and the Scientific Community (local, national and international).

The dissemination of the project findings and datasets, via open access publication will have significant impact on the wider scientific community, in terms of both knowledge transfer and best practice.

<u>Patients and the NHS.</u>

The outcomes of this research will likely lead to the development of new approaches and tools to treat intestinal diseases. Long-term, this is likely to have significant health impacts on specific patient groups by improving quality of life and health. More generally, the translation of this preclinical research into treatment of gut motility disorders may have significant benefits to the NHS: both economically and in terms of provision, and delivery, of services. To date, gut motility disorders have been very difficult to treat. Current treatments are limited to long-term drug management of symptoms or in many cases surgical intervention including bowel transplant with lifelong immunosuppression as a worst-case scenario. However, such treatments are associated with poor outcomes and significant long-term issues. Often patients require life-long drug treatment or long-term intravenous nutrition dependency and multiple hospital admissions which are costly to the NHS and wider UK economy. The outcomes of this research will increase our understanding of these complex diseases and will potentially provide therapeutic advancements to treat these devastating diseases. This is likely to have long-term indirect benefits on NHS efficiency, via reduced hospital admissions/long-term prescription uptake.

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

In order to maximise project outputs all project data will be made available to interested researchers via open access publication in peer reviewed journals. Our approach to data sharing will be outlined in any, and all, publications resulting from this work and will

conform with specific peer-reviewed journal guidelines. Datasets (e.g., single cell RNA sequencing) held outside of the institution (e.g., Gene Expression Omnibus) will be publicly available and discoverable.

Additionally, to benefit the research team, and wider scientific field in general, regular dissemination of project work will be performed via conference participation and the development of collaborative work with other leaders in the field of neurogastroenterolgy. Further, our institute regularly engages the public with its research and innovation through public lectures, podcasts and participation in open days. Any findings of the current research will be communicated with the institutional media teams to maximise the reach and impact of the research programme.

Species and numbers of animals expected to be used

- Mice: 10,000

Predicted harms

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

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

We plan to use mice (embryos to adult) to investigate gut development, disease and regenerative medicine with specific focus on the development/dysfunction/regeneration of the gut nervous system and associated cells (smooth muscle, ICC and myofibroblasts) within the gut wall. The mechanisms that control these key developmental and disease processes are poorly understood but are known to include cell-intrinsic factors, in addition to interactions with other cells and tissues as the gut develops. Such complex interactions between cells and their environment cannot be mimicked by cells grown in vitro, therefore there is no alternative other than to investigate these processes within animal models. Moreover, multiple mouse models exist which show similar gut disease symptoms to that of humans allowing direct study of gut development and disease which can be translated to human-beings.

We also plan to use adult mice in order to assess whether donor stem cells from various sources (e.g., gut nervous system stem cells) can be used to replace missing or defective gut cells in disease states. As we hope to develop possible treatments that could potentially help people, we first need to test how any donor cells: (i) survive, (ii) integrate into the appropriate locations in the gut, (iii) form the correct cells, (iv) form functional connections with the surrounding gut cells and (v) ultimately restore gut function. Therefore, in vivo use of adult mice for such studies is required as current alternatives (e.g., tissue culture), although useful, cannot mimic the complexity of functioning organs, let alone the entire body.

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

Typically, mice used for specific transplantation studies, to determine if it is possible to treat gut disease with cell therapy, will receive the most procedures. Here, mice will typically be bred with a specific gut disease (e.g., Hirschsprung disease, constipation). At approximately 2-4 weeks of age, we will perform surgery on these mice to transplant donor cells into the gut wall. These surgeries on average take approx. 20-30 mins. Mice will then

be treated daily, for up to 5 days, with pain relief injections under the supervision of an NVS. At various timepoints after transplantation we will then assess the mice to determine whether our cell therapy has been successful. Typically, this will be done at 4 weeks post-surgery, but longer times (e.g., up to 12 months) may be required to assess the safety of our treatment. To assess gut function, we will feed the mice a dye and assess how this is transported through the gut. These functional assays take approx. 2-3 hours.

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

Animals with a genetic modification resulting in a gut motility disorder may show clinical signs including development of slow intestinal transit for the duration of their lifespan. Mice undergoing surgical procedures are expected to experience mild-moderate pain in the immediate aftermath of the surgery. Mice will be given pain relief for a period of up to 5 days post-surgery under the supervision of the NVS. Such mice may also experience some mild weight loss for several days following surgical procedures. However, close monitoring, including weighing of animals, and administration of food substitutes for 7-10 days following any surgical procedure will be used to minimise any potential weight loss in these 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 vast majority of animals on this project are expected to experience mild severity (70%) including the majority of breeding under this licence. The remaining 30% are expected to fall under moderate severity protocols including protocols which will disrupt the cellular constituents of the gut wall, by chemical or physical means, and/or mice that will undergo surgical transplantation of donor cells.

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

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 plan to use mice (embryos to adult) to investigate gut development, disease and regenerative medicine with specific focus on the development/dysfunction/regeneration of the gut nervous system and associated cells (smooth muscle, ICC and myofibroblasts) within the gut wall. The mechanisms that control these key developmental and disease processes are poorly understood but are known to include cell-intrinsic factors, in addition to interactions with other cells and tissues as the gut develops. Such complex interactions between cells and their environment cannot be mimicked by cells grown in vitro, therefore there is no definitive alternative other than to investigate these processes within animal models.

We also plan to use adult mice to assess whether donor stem cells from various sources (e.g., gut nervous system stem cells) can be used to replace missing or defective gut cells in disease states. As we hope to develop treatments that could potentially help people, we first need to test how any donor cells: (i) survive, (ii) integrate into the appropriate locations in the gut, (iii) form the correct cells, (iv) form functional connections with the surrounding gut cells and (v) ultimately restore gut function.

Therefore, in vivo use of adult mice for such studies is required as current alternatives, although useful, cannot mimic the complexity of functioning organs, let alone the entire body which will be crucial for characterizing the safety of any cell therapy.

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

The use of human embryonic gut tissue (obtained from the Human Developmental Biology Resource) and patient material (obtained with fully informed consent), which would otherwise be discarded.

Cell/Organoid culture

Why were they not suitable?

The use of human material will be utilised as a replacement where possible (i.e., ex vivo transplantation studies in human discarded tissue) and scientifically appropriate. Unfortunately, the use of human embryonic gut tissue is necessarily restricted to descriptive studies of human development and disease at any specific time point. While these are informative, they lack the key ability to investigate dynamic interactions of different cell types with the gut wall. Further, experimentation in human discard material is, by definition, limited to tissue which may not be ideal to address a specific question. In addition, such human tissue cannot be cultured for extended periods, outside of the body, limiting our ability to test the long-term effects of cell therapies. Moreover, given our aim of investigating development and disease processes in gut motility disorders: the ability to perform comparative studies in control tissues is vital for quantitative hypothesis testing. Unfortunately, in terms of childhood gut disease, human control tissues are extremely difficult to obtain, again restricting studies to mainly descriptive findings.

We will use cell/organoid culture for cellular studies: where analysis of whole embryos/tissues is not essential. Although these techniques can provide useful information on certain specific molecular or cellular phenomena, they cannot mimic the complexity of functioning organs, let alone the entire body. For example, gut organoid culture has progressed significantly within the last 5 years however key components of the gut wall (i.e., interstitial cells of Cajal) fail to develop in these structures in vitro and are only observed following in vivo transplantation of gut organoids. It remains unclear as to why this phenomenon occurs. However, such findings limit the applicability of in vitro organoid applications for the current research which seeks specifically to understand the interaction of different cell types in the development of the gut and their potential for regenerative medicine 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?

The numbers estimated are based on previous experience including mouse colony management and prior experience in the numbers of animals required for developmental/transplantation studies to ensure reproducibility and statistical robustness. Further, discussions with a statistician were used to extrapolate these estimated numbers to include enough animals, of the correct genotypes, required for these studies including controls which will consider the sex, age and disease state of any mice used.

To maximise the data gathered from any individual animal multiple biological samples (i.e., different regions of the gut or other organs/tissue) will be collected from experimental animals where possible. We will also utilise our extensive network of collaborators to share any tissues that may be of interest to others studying other organ systems.

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

For all in vivo experimentation, the use of the NC3Rs EDA has been/will be utilised to ensure optimised experimental design and reduce animal usage. For example, the EDA has been instrumental in streamlining previous in vivo transplantation studies to ensure i) sufficient animals and controls have been used to provide statistical power and reproducibility and ii) reduce overall animal usage to the minimum number required for this. Further, ongoing discussions with an appropriate NACWO and NVS have been utilised to ensure a minimum number of animals are used where multiple protocols are required. These discussions have been critical in the design of experiments in accordance with the 3Rs principles while maximising experimental data and animal welfare.

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

Methods for optimising the overall numbers of animals used in this project include the use of:

In vitro culture as a primary assessment of gut stem cell behaviour. Prior to use in animals, we will optimise and characterise gut stem cell behaviour in vitro, thus reducing the number of animals used.

A limited number of animals used for in vivo analyses (e.g., 6-12 animals, sufficient to ensure reproducibility).

Ex vivo transplantation techniques. Our lab has recently pioneered to use of a novel ex vivo gut transplantation technique which reduces the number of animals required for transplantation studies by >84%. Where appropriate, initial transplantation of donor cells will be performed to ex vivo gut segments as standard to reduce animal numbers 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.

Within this project we aim to use mice (embryonic to adult) to investigate gut development, disease and regenerative medicine approaches.

This research project makes extensive use of genetically modified mouse strains to enable studies on gut development and disease with the ultimate goal of developing regenerative medicine approaches to better treat human gut disorders. No other animal model offers the combination of the practicality of a laboratory model, the availability of a number of genetic models of gut defects and accessibility for
transplantation of gut stem cells as a restorative therapeutic approach. Importantly, genetically altered mice offer the most incisive approach to the analysis of gut defects mechanisms, because:

Mouse genetics is understood almost as well as in humans, offering the best possible means for genetic analysis in a mammalian organism.

Gut disease in genetically predisposed mice closely resemble those found in human patients, providing excellent models for analysis of disease mechanisms and for evaluation of preventive/restorative treatments.

Transgenic/gene knockout technologies in mice offer a sophisticated method for studying the mechanistic effects of specific gene mutations in particular tissues, or at specific life stages.

The vast majority of genetically altered mice used in this project will fall under mild severity protocols due to mild phenotypes associated with heterozygous genetic alteration, or mildly affected homozygote states, depending on the gene of interest. Such genetic manipulation offers refinement over traditional chemically induced models (e.g., 2,4,6-trinitrobenzene sulfonic acid (TNBS) administration) which often leads to unwanted off-target disruption of multiple cell types in the gut wall: leading to unnecessary suffering and lasting harm.

In the small number of experiments involving moderate severity protocols (e.g., in vivo transplantation of gut stem cells) the focus of the study is to test treatment for rescue of the phenotype (e.g., to correct for the lack of cells within specific regions of the gut) or better understand the mechanism of disease. In such studies, we will utilise mice at early timepoints (i.e., prior to the onset of gut obstruction/dysfunction) as recipients for stem cell injections and studies incorporating partial gut disruption will be designed to ensure damage to the gut is reversible and continuity of the gut is maintained at all times. Further, where in vivo surgical manipulation is required, appropriate protocols for anaesthesia and post-operative analgesia will be used: in close consultation with an NVS and NACWO, to ensure to minimise pain, suffering, distress and lasting harm. We do not intend to pursue any severe severity protocols.

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

Pre-clinical models that faithfully replicate the pathobiology of childhood and adult gut motility disorders disease are not available in less sentient animals. Many of these diseases occur at postnatal or adult life stages so using animals at a more immature life stage is not suitable. Animals that are terminally anaesthetised are not suitable for our work as this does not provide an opportunity to observe disease progression, pathophysiology or examine the impact of therapeutics on potential rescue of gut function.

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

Where possible, we will refine procedures to i) eliminate the need for certain moderate severity procedures (i.e., in vivo transplantation) and ii) reduce the number of animals used for specific transplantation studies by utilising the novel ex vivo approach developed within the lab.

Where required, we will seek ongoing engagement and advice from both the appropriate NACWO, NVS and Home Office inspector to refine post operative management. Such engagement has, to date, led to significant alterations in analgesic regimes in previous studies. Further, any mouse that has undergone a procedure will be monitored closely by research staff as well as daily by Biological Services staff with input from the appropriate NACWO. Any mouse undergoing a surgical procedure will be closely monitored and provided post operative care on a daily basis, for a minimum of 5 days, by both research staff and designated animal technicians including the completion of written records of all monitoring (e.g., weight, body condition, incision monitoring etc).

To further refine procedures, where possible, oral administration of drugs will be performed as opposed to injection (e.g., oral administration of tamoxifen vs IP injection) to minimise welfare impacts on any experimental animals.

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

We will refer to the most updated LASA (http://www.lasa.co.uk/publications/) and PREPARE guidelines (Laboratory Animals 2018, Vol. 52(2) 135–141) when planning each new study. We will also utilise the NC3Rs Experimental Design Assistant resource to ensure robust design of experiments. For more specific best practice guidance (e.g., administration routes/volumes etc) we will refer to published guidance (e.g., LASA, EFPIA and ECVAM) to ensure experimental procedures are conducted in the most refined way and adhere to best-in-class practice. Furthermore, experiments will be conducted in a manner to enable open access reporting in accordance with the ARRIVE 2.0 guidelines to ensure reproducibility.

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

Regular project-specific discussions between researchers and animal technicians, the relevant NACWO and NVS will occur, throughout the duration of this project, to review current and new 3Rs approaches. Project staff will ensure latest 3Rs developments are kept up-to-date by engaging with the NC3Rs (i.e., monthly e-newsletter) and the local NC3Rs representative (i.e., attend local events and workshops). Further, we will refer to the NC3Rs website https://nc3rs.org.uk/staying-informed-latest- 3rsadvances and the most updated LASA guidelines (http://www.lasa.co.uk/publications/) on techniques relevant to

this project (e.g., LASA 2017 Guiding Principles for Preparing for and Undertaking Aseptic Surgery. A report by the LASA Education, Training and Ethics section. (E Lilley and M. Berdoy eds.) for additional information and we will ensure early and frequent interaction with the NC3Rs in any future grant applications which result from this project licence. Ongoing studies (funded by NC3Rs), following such engagement, have allowed reduction in the number of mice required for transplantation studies by application of a novel ex vivo organotypic approach.

9. Immunity, pathogenesis and transmission of Culicoides-borne viruses of ruminants

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Viruses, Insects, Transmission, Vaccination, Control

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The overall aims of this project are to contribute to developing control and prevention measures against those viruses spread between animals by the biting flies, *Culicoides* biting midges. Protection of the individual ruminant host against midge-borne viruses is either achieved through immunisation of individual animals or by protecting the ruminant population through disruption of virus transmission by biting insects.

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

Why is it important to undertake this work?

Some viruses do not spread directly between their human or animal hosts but instead

infect blood- feeding insects or ticks, which then pass the virus on during feeding. In Europe, the most important animal viruses transmitted by insects are those spread by Culicoides biting midges, which infect ruminants such as cattle, sheep, goats and respective wildlife. Of midge-borne viruses, Bluetongue virus (BTV) poses the biggest risk to ruminants and in outbreaks has already caused substantial animal suffering and economic losses of millions of Euros in many European countries. The costs associated with the 2006-2010 BTV-8 outbreak have been estimated at 180 million Euros in Germany alone. Other midge-borne viruses such as epizootic haemorrhagic disease virus (EHDV), Schmallenberg virus (SBV) or bovine ephemeral fever virus (BEFV) have also either recently occurred in Europe or are considered potential risks as they are transmitted by the same insects.

Viruses transmitted by insects are very difficult to control as Culicoides biting midges are very small and infected insects can fly or be blown vast distances.

Within this license we want to address key knowledge gaps to develop better control measures so that either less ruminants become infected by midge-borne viruses or less ruminants develop painful disease. Our objectives are to gain a fundamental understanding of the immune response of animals to BTV vaccination and the factors that determine virulence and onwards transmission of midge-borne viruses.

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

Control measures against midge-borne viruses are carried out through two principles: Protecting the individual mammalian hosts from infection through vaccination (of specific importance for BTV) and disrupting or preventing virus transmission by the insect vectors thereby protecting the population of mammalian hosts. The objectives of this licence address both of these principles.

Studies undertaken in this licence will allow us to identify why a proportion of cattle do not appear to develop detectable BTV antibodies after vaccination even when tested across multiple assays. We hope to establish indicators of appropriate vaccination status, thereby reducing risks associated with importation/trade of these animals from regions where BTV circulates and animals are vaccinated.

Additionally, the study will enable us to compare cattle immune responses following BTV vaccination to those previously identified in ruminants during BTV infection, thereby generating further insight into whether immune responses to BTV infection and vaccination differ and identify potential future opportunities to improve BTV vaccines.

In addition, our studies will aim to identify virulence mechanisms of midge-borne viruses such as BTV and factors driving transmission to and from blood-feeding insects.

It is currently impossible to determine virulence, pathogenicity and transmissibility traits of midge-borne viruses (specifically across different BTV strains) in vitro as they involve multiple interactions within the host. It is important to investigate phenotypic differences of BTV and EHDV strains, including those that represent an emerging threat to the UK livestock industry. Assessing phenotypic characterisations of viral strains will also greatly help to identify the genetic basis of virus virulence and transmissibility.

Determining the efficiency of virus transmission to and from insect vectors and ruminant hosts is key across the midge-borne viruses (BTV, BEFV, EHDV and SBV) will enable the implementation of control measures that aim to reduce viral spread. Determining the time

frame of infectiousness of ruminant hosts to blood-feeding insects and its relationship to detectable systemic viremia in the host is critical to be able to predict viral spread by mathematical modelling which might influence vaccine deployment, removal and protection of individual ruminants and the implementation of control zones.

Data from animal studies contextualise data from artificial feeding of insects where the impact of the immune response on infectivity of virus within the host cannot be replicated.

We will publish the data sets acquired through the outlined experiments in open-access, peer reviewed journals. Further dissemination of the knowledge gained will be achieved through presentations of the work at scientific conferences, interest groups and specific disease policy meetings.

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

Results obtained in studies under this licence will be used by policy makers, the livestock agriculture sector, the wider veterinary and medical arbovirology research community and might influence product development in the long term. Data from immune response to vaccination and pathogenicity and transmissibility of exotic midge-borne viruses will be of direct benefit to policy makers (Defra) in risk assessments of incursion and mitigation prior to and during an outbreak in the UK. This will be communicated directly with the Department of science and policy leads through study reviews, established contacts and disease expert groups. Many of the activities performed under the authority of this licence will be highly relevant not only for arboviral diseases of livestock but also to those of humans e.g. Zika virus. Human arboviruses often need to be studied in less relevant rodent models while research into ruminant arboviruses has the benefit of being investigated in its natural host.

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

Outputs will be maximised through publication in open access journals. Our network of collaborators and interested parties, through the vector-borne disease research community and National Reference Laboratory activities will be used to promote our work to a national and international audience.

Species and numbers of animals expected to be used

- Cattle: 240
- Sheep: 60

Predicted harms

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

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

Cattle and sheep represent the host species of the midge-borne viruses to be studied. As such they represent the natural animal model for investigations of these viruses, providing data that is directly relevant to the field. In vaccination studies animals of sizes representative of UK herd composition will be used. In transmission experiments, cattle will be used as young animals of less than 120 kg on receipt for ease of handling in our animal

facilities. Adult female sheep typically demonstrate greater clinical disease than younger animals and ewes at the end of reproductive productivity will therefore be used in most studies.

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

All animals will have blood samples taken on farm to screen for virus-specific antibodies and suitability for our studies.

Vaccination and immunology: Animals may be vaccinated with a commercial BTV vaccine to assess immune responses. Responses will be compared to those reported in previous, virus infection studies. Blood samples will be taken throughout the duration of the project at time points up to a year from vaccination. Vaccinated animals will be returned to their herd following the experiment.

Virus infection: Animals may be infected with a midge-borne virus, primarily through the bites of infected Culicoides midge vectors or alternatively through subcutaneous needle inoculation. Blood samples will be taken throughout the experiment. Further exposure to the feeding of naïve Culicoides biting midges on animals may take place at intervals through the infection cycle. Animals in infection/transmission trials will be euthanized at scientific or humane endpoints, if reached.

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

Vaccination and immunity:

The vaccination used under this licence will not result in any adverse reactions, except possibly a local reaction at the injection site such as erythema and/or swelling. Systemic allergic responses to vaccination are rare but a mild and transient rise in body temperature might occur following vaccinations.

Virus infection:

Of the midge-borne viruses used under this licence (BTV, EHDV, SBV and BEFV), pronounced clinical disease is only expected for BTV infections of sheep. Disease will be determined by the inherent virulence of the most virulent virus strains used.

Typical signs of BT in sheep develop normally between 5-14 days post infection and are characterised by: rises in body temperature resulting in temperatures classed as fever, reddening of mucosal membranes occurs, including the conjunctiva of the eye. Ulcers and small petechial bleedings may occur on the nose, gum and dental pads. Subcutaneous oedema may develop in the face and lips.

There may also be inflammation and reddening of the coronary bands possibly causing some lameness. Nasal discharge and excess salivation may occur.
Sheep infected with EHDV are mostly asymptomatic, however should clinical signs develop these would mirror a BTV infection.

Cattle very rarely show clinical disease to infections with BTV or EHDV. Occasionally they may show a rise in body temperature. Highly virulent strains of BTV or EHDV may rarely lead to similar clinical signs as described for sheep.

Cattle may develop mild disease to BEFV infections namely fever, depression, reluctance to move and salivation.

SBV infections of adult ruminants are either clinically unapparent or mild with raised body temperature and diarrhoea.

Local responses to arthropod blood-feeding vary greatly between individual animals and range from small petechia, typical bite nodules to a substantial local oedemic inflammatory response.

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

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

Vaccination and immunity: Mild severity, 100% of animals. Clinical impact from vaccination and repeated blood sampling is expected to be transient and mild.
From previous studies with BTV infection we expect: 50% of infected sheep to experience moderate severity, with 50% experiencing mild severity. In cattle, we expect 100% to experience mild severity. Animals will be euthanised prior to the development of pronounced disease, but are likely to exhibit clinical signs of BTV or EHDV infection. All animals within each trial may be infected, with the exception of contact transmission controls, if required.

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

- Killed
- Rehomed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.
Why do you need to use animals to achieve the aim of your project?

It is extremely challenging to examine the complex interactions between mammalian hosts, insect vectors and transmitted viruses experimentally and not possible to achieve *in vitro*.

One of the main aims of this work is to gain a fundamental understanding of the role of the immune response in resistance to and pathogenesis of midge-borne viruses in order to improve and develop vaccines and prophylactic and therapeutic strategies. The immune system is highly complex and inter- related and an understanding of the immune response in infectious disease requires the use of living animals. Additionally, our current knowledge of the immune response of ruminants to these viruses does not allow protection afforded by different vaccination strategies to be predicted. In alignment with other research groups, the long-term goal is to identify measurable correlates of protection and so reduce the need to perform challenge studies with virulent pathogens in the future.
Furthermore, it is currently not possible to assess the virulence of emerging orbivirus strains in vitro or within a mouse model system. Several studies investigating the virulence of genetically modified viruses have demonstrated that phenotypic behaviour of specific viruses in cell culture is not correlated with replication characteristics in mice, which in turn

does not reflect the true pathogenicity observed in ruminants. Additionally, a complete biological system is required in order to study transmission, especially for vector-borne pathogens where successful transmission requires the interaction between vertebrate hosts, blood-feeding arthropod vectors and the pathogens. Therefore in vivo studies have to be performed in order to be able to elucidate transmission mechanisms, risk periods and efficiencies.

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

We are investigating if ruminant host derived organ explants and/or primary cell cultures can be used to reflect the phenotype of these viruses in the future. These techniques could be used to study pathogenesis and specific cellular responses to arboviruses and arthropod saliva, potentially aiding in the development of genetic markers for virulence or transmissibility.

Why were they not suitable?

Use of primary cell cultures and organ explants for determination of virus phenotype is at a very early stage of development and has not yet been able to provide the data required for these 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?

For the vaccination studies, the number of cattle was chosen: (i) to be similar to a UK dairy herd, so the range of responses post vaccination would likely be similar to that observed in the field; and (ii) to make sure there would be sufficient animals present throughout the duration of the study, so not too many would be lost through turn-over as part of routine herd management. The aim of the study is to construct typical profiles of how a range of immune parameters change with time post vaccination which may subsequently be used to infer the vaccination status of an animal and when it was likely to have been vaccinated.

For transmission studies where we aim to characterise pathogenicity and transmission parameters of a virus strain, fewer animals are required. We rely on our previous, similar studies, where a treatment group of 6-7 individuals was sufficient to capture the range of individual response to infection. Our infection protocol has provided very high efficiency of infection and therefore we are confident of all animals exposed in studies exhibiting infection and viraemia, reducing the need for additional animals to ensure infection.

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

Specialist statistical advice is a critical element of our animal experimental design. In the past decade we have built up a significant data catalogue which we use to estimate variation between animals in response variables and inform appropriate effect sizes for

use in power calculations. The numbers of animals used in experiments will be the minimum possible to achieve statistically robust data (typically we will aim to detect differences with 80% power and 95% confidence), but previous comparable studies have been between 7-9 animals per treatment group depending on most variable outcome used for power analysis. For example, groups of 8 sheep were used to successfully characterise differences in pathogenicity of a re-emergent strain of BTV -8 in sheep. While each experiment will require the inclusion of appropriate controls, these will be kept to the minimum that does not jeopardise the reliability or integrity of the experiment.

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

Initial proof-of-principle studies will be carried out on a smaller number of animals in those studies were this is appropriate. These proof of principle studies will then be used to inform power calculations used to determine animal numbers for follow–up studies, while unsuccessful proof-of principle studies might result in the aim not being investigated any further.

The phenotypic behaviour will firstly always be investigated in insect-derived cell cultures and subsequently in adult vector insects. Only once specific replication characteristics within the insect vector have been confirmed (low or high replication efficiency) will the transmission sequence between ruminant host and insect vector studied in vivo.

Refinement

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

Which animal models and methods 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 perceive it as a specific strength that the studies into the immune response and pathogenesis of midge-borne viruses proposed under this licence will be carried out in ruminants, the natural host species. Most experimental studies of vector-borne viruses utilise either needle-inoculation of the virus into the host and/or the use of non-natural model species such as rodents. However, we are able to transmit the midge-borne viruses directly between the natural ruminant host and relevant blood feeding insects, an experimental design that is exceptionally representative of natural infection.

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

While a mouse model of clinical bluetongue has been developed using mice deficient in a major antiviral mechanism, striking differences between the murine and the ruminant immune system and the deletion of an important anti-viral mechanism renders such mice unsuitable for our studies.

Additionally, arthropod feeding on small rodents is likely to result in different cell migration dynamics partly due to the small size of the animals. For example, in certain studies of

mosquito-borne arboviruses it was demonstrated that feeding of infected arthropods on mice would immediately result in detectable viraemia simply due to the small blood volume. Time course feeding of arthropods through the cycle of infection means that animals under terminal anaesthesia cannot be used.

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

Through the experience gained under previous Project licences, specific scientific endpoints have been designed for protocols which will significantly reduce the impact on the animal. Animals in virus characterization studies will be euthanized upon confirmation of viraemia and initiation of clinical signs of disease rather than development of pronounced disease. Throughout previous projects the blood- feeding of insects on cattle and sheep has been continuously modified. Blood-feeding success rates of exposed insects on sheep is highest when allowed to feed on the inner thigh. However only 10 minutes exposure results in high feeding rates and only very rarely are longer exposure times (max. 30 minutes) on sheep required. On cattle, insects need longer to successfully blood-feed presumably due to the thicker skin. However, here insects feed best on the rump, neck or side which allows cattle to be minimally restrained in a feeding head-lock. Therefore, cattle exposed to blood-feeding insects for an average of 20 min (max 30 min) do not display any aversion or stress behaviour.

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

The Laboratory Animal Science Association (LASA) guidance will be consulted prior to each experiment to ensure best practice is known and adhered to.

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

Guidance on 3Rs implementation will be reviewed prior to each animal experiment. Science investigators will explore the literature published by other groups working with BTV and communicate with animal unit staff to ensure each is updated with relevant developments within the type of studies undertaken.

10. Immunological defense against pathogens and physiological consequences of infection

Project duration

5 years 0 months

Project purpose

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

Key words

Infection, bacteria, virus, lung, health

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The first aim of this project is to determine how the immune response protects our bodies against pathogens and how this response changes the way our bodies work during infection and disease. The second aim is to change the way the immune response develops to increase protection against pathogens while reducing the damage that the immune response does to our bodies during infection and disease.

Our work will allow us to develop interventions such as vaccines, drugs and lifestyle changes and to also develop diagnostic and predictive tools to reduce the impact of infection and disease in people.

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

Why is it important to undertake this work?

Infections, and the diseases they cause, still claim many lives and reduce our health and well-being. We have recently experienced how devastating a new virus can be and we are still suffering many deaths and illness as a result of diseases such as tuberculosis and AIDS. Whilst we have made great progress in developing vaccines against diseases that can be controlled by antibodies, we still have no effective vaccines against many diseases which require complex immune responses. Recent evidence from the pandemic also shows us that the consequences of infection and disease can last past the time when the bacteria or virus resides in our bodies. Understanding how the immune response that protects us also alters our body's functions is important to allow us to develop interventions that can reduce both the short and long lived consequences of infection and disease.

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

We will be investigating the pathways by which our immune response stops bacteria and virus growing. We will also be determining how our bodies respond to the infection by changing how our organs work and how this affects our well-being. The work that we perform and the information we collect from our work will be guided by our clinical colleagues who work with infected people and who study both the immune response and disease in these people. We will combine our knowledge with that of our clinical colleagues and write documents that are communicated to other scientists and clinicians, which will then help us to develop and improve working models of disease and thereby drive changes in how we manage these diseases. We have been working on developing vaccines, diagnostic and prognostic tools and will continue to improve their design and relevance by comparing the outcomes we see in the animal models with the data we collect from our human experimental medicine studies.

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

We expect that the primary beneficiaries will be those people who are focused on improving the control of infection and disease in people. The information that we generate will allow them better understanding of how human symptoms result from different immune mechanisms occurring when an immune response protects us from bacteria or virus infection. The information we generate will help in the design of vaccines to initiate strong immune responses that do not damage the person and effectively control the bacteria or virus. The information we generate will also help those who are developing diagnostic and prognostic tools to manage individual patients. Some outcomes could be delivered quite quickly if we show that early bacterial infection results in altered sugar biology in the infected animal then we could rapidly implement sugar biology monitoring tools during specific infections in the clinic.

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

We are part of several collaborative endeavours both within the UK and Europe. These collaborations are with clinical groups, vaccine developers, industrial partners looking at new drugs and those looking to understand the impact of infection on health. We share knowledge and actively collaborate to ensure maximum benefit to the public while limiting harm to the animals. We publish our scientific papers together, we meet together as mixed groups of scientist, clinicians, and industry partners and share the long view of what we are trying to achieve.

Species and numbers of animals expected to be used

- Mice: 9600

Predicted harms

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

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

We have chosen mice as they have a defined genetic makeup; they can be housed in conditions that maintain health and well being; there are extensive tools allowing very detailed monitoring of immune and health responses; they can be manipulated to alter the expression of specific molecules on specific cells or tissues over time. This manipulation allows very detailed understanding of the role of these specific molecules, cells and pathways over time. We have chosen mice from birth to adult stages to ensure we understand how infection and disease impact health over the lifespan.

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

Both genetically modified (GMO) and non GMO mice can be bred in house or obtained from external appropriate sources. Some mice may be handled in the first few weeks after birth to receive substances that will influence their immune responses later in life. Other young mice may have tissue taken to undertake genetic identification. Most mice will reach early adulthood without intervention and will then receive an intervention such as a vaccination, cells, or drugs that will change their immune response. These interventions will be by an approved route and dosing schedule such as injection through the blood, muscle, under the skin or into the abdominal space or via breathing (in the air), water or food. These interventions may occur more than once but not more than allowed by the approved guidelines. Some mice that have received an intervention can then be killed and the response to the intervention measured. Other mice that have received an intervention will then also be infected with a bacterium or a virus via an approved route (blood, skin, tissue, lung). Some mice will either have no intervention or a sham intervention before infection and these serve as control mice allowing the impact of the intervention to be measured. After infection, the mice are monitored via measures of health - this can be weight, appearance, behaviour - using a defined scoring scheme.

Some mice may be fasted (i.e. food removed for a period of time) and may receive an injection of a substance to allow us to measure the effect on body functions such as sugar and fat biology, muscle function and other measures of wellbeing. Some mice will receive a special diet to alter the level of nutrients within the body. All mice will eventually be killed and measures of immune response and general health taken. A few mice may be irradiated and receive cells to reconstitute their blood cells, these mice will receive only one or two interventions/infections to reduce the total harm. Most of the mice will receive an intervention and an infection and may undergo blood sampling after infection. The infection models do not require mice to become severely ill and the scoring systems and monitoring we use is designed to determine how ill the mice are and allow us to kill them before they suffer anything more than moderate harms.

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

Most injections and interventions will cause momentary pain during the time of intervention. If more substantial but short lived pain is expected then analgesia will be provided prior to and during the pain event. The infection process should not be painful and the infection models we use will largely result in chronic low level of infection. Progression from a low level of infection to a more diseased state will be monitored by weight loss, appearance and behaviour using well defined scoring system. The scoring system and frequency of monitoring mean result in defined cut off where mice are killed to ensure that they do not suffer beyond moderate levels. There is no benefit to the studies we are performing of the mice reaching a severe level of harm. Viral infections are usually shorter than bacterial infections but as long as mice appear healthy they may be infected for prolonged periods, which allows us to understand the long term effects of chronic low level infection on the body.

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

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

Most mice will have low level of suffering i.e. short lived pain from injection and little impact of the infection. Some mice may undergo moderate suffering i.e. those receiving an irradiating dose to remove their blood cells. Some mice may have a moderate level of suffering due to fasting followed by injections and being bled more than once in a day. Some mice may undergo moderate suffering as a result of infection and disease development but this is expected to be short-lived due to the monitoring and scoring mechanism. Approximately 80% of the mice will have mild or short term moderate suffering with 20% experiencing longer term mild to moderate suffering - not to reach the extent of severe suffering.

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

- Killed
- Used in other projects

Replacement

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

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

Animals provide a complex system of interacting organs and systems wherein many unknown unknowns contribute to the outcome. The events and interactions we want to measure - i.e. determining how infection impacts the well being of the whole animal cannot be achieved with in vitro of ex vivo organoid systems. Because mice can be manipulated at the gene level we can investigate the role of single molecules in driving long term and complex events in a definitive and non-ambiguous manner.

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

We use clinical outcomes and experimental medicine data to formulate the experimental

activities we undertake in mice. We also use integration of large data sets to identify potential pathways and to avoid repeating experiments already undertaken. Structural biology and pharmaceutical data sets are also used to ensure focused and meaningful experiments are designed. Some cell work in the lab is also undertaken to determine the types of cells and molecules that may contribute to the outcomes and to investigate signaling pathways. We engage with colleagues and the literature to follow development of 3-D culture and organ-chip models to ensure we use these resources to refine the questions we address in mice.

Why were they not suitable?

Our laboratory takes an integrated approach using all available tools to ensure we determine the molecular and cellular pathways that act to cause disease. We can neither deliver interventions and infections at a defined dose and defined time, nor genetically manipulate humans and so we use mice as a model vertebrate. We cannot ensure that cells and systems are working as designed when we culture cells in the laboratory and therefore we need to use the mouse as an integrated well designed vertebrate model that can faithfully report the cellular and molecular pathways that can occur in a complex system.

Reduction

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

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

We have data sets generated in the past showing that the information we receive from mouse experiments does not vary much between in-bred mice, as the mice are very similar genetically and they have very similar life experience. This means that we can use quite small numbers of individual mice within each experimental group - generally 5 mice per group. We will usually try to use control (1) mice across a number of experimental groups - probably about 3 for anyone experiment ((1+3) groups x 5 per group = 20). There will generally be 3-8 timepoints to account for early, mid and late events during infection and disease (4 groups x 5 per group x 5 time points = 100), thus we might use 100 mice for a full experiment. For some experiments the number of time points may be reduced. We will also be breeding mice and this is a variable number depending upon the number of mice required for experiments - some genetically manipulated mice have 4-5 genes that we need to cross for and while most mice without the right genes can act as controls there are some that cannot be used. Most of our colonies have 3-5 breeding sets - 2 females 1 male = 3 x 5 sets = 15 breeding mice per colony, which are renewed every 3-6 months. All breeding colonies are monitored and genetically typed rapidly to avoid generating mice that cannot be used.

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

We use the Experimental Design assistant to help us ensure that the experiments we perform will be robust, reproducible and meaningful without excess use of mice.

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

We will use control mice across a variety of experimental groups thereby reducing animal numbers. If we find during our studies that the data does not require as many as 5 mice per group we will reduce the number. We will not perform experiments that have been performed before. It is now possible to access the raw data from other laboratories work and we can use that to ensure we are not repeating 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.

We use mice because they can be housed and handled in an environment that is not stressful for them. They can be housed together, they can build nests, they have social interactions and can be acclimatized to their environment. We will use the least invasive way to deliver any intervention or infection. The aerosol route - which is our speciality - consists of placing the mice in a large chamber and allowing them to move about in a cloud containing the bacteria or virus.

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

We are focused on how infection in the vertebrate lung changes the way the rest of the body works. We cannot use other animal models for this as the differentiation of the lungs and the compartmentalization of the rest of the body is fundamentally different. We are also looking at the changes in the response of the body over time so we need to keep the animals alive. It is important to our work that the animals remain largely healthy and not stressed so that we are only measuring the impact of infection on the body - rather than the stress of profound disease or pain. Our scientific goals will be compromised if the animals become distressed.

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

We have experienced and engaged animal caretakers who are part of the scientific team. We discuss with the caretakers why we are preforming the work this encourages intellectual engagement with the monitoring and well being of the mice. We make use of the experienced staff within the animal facilities to advise and support our laboratory staff members to ensure our monitoring schedule and health scoring sheets are up to date. The scientific goals of the experiments can be compromised if mice are stressed or feeling pain and we monitor for altered behaviour, altered condition and weigh mice frequently to ensure they are not undergoing long term stress.

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

We engage with the refinement literature and on site and on line presentations in an ongoing manner. Statistical and experimental tools that reduce numbers, stress or pain will be implemented throughout the course of the project. We follow the PREPARE and ARRIVE guidelines and report the use of these in our publications.

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

We have a close relationship with the NC3Rs and the Establishment licence holder is committed to ensuring all staff working with animals fully engage with the 3Rs - it is the fundamental underpinning of how we approach animal work within the establishment. As project licence holder I will continue to be engaged and will ensure all staff working for me remain engaged - allowing time and training opportunities whenever they become available.

11. Interaction between the immune system and tumours

Project duration

5 years 0 months

Project purpose

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

Key words

Cancer, immune cells, gene, immunotherapy, function

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

To understand how genes (which are the key instructions inside the body) within the cells of the immune system (that normally helps protect the body against infections) can effect the way in which these immune cells interact with cancer cells. This can result in an alteration to tumour growth or spread or the direct killing of cancer cells. This will also include studies on how particular aspects of cancer cells can affect the function of immune cells within tumours.

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

Why is it important to undertake this work?

It is now predicted that 1 in 2 people will develop cancer in their lifetime and despite advances with new treatments they do not work for everyone. By providing further

understanding how the immune system can detect tumours and eliminate cancer cells it will provide new avenues for treatments.

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

We will generate a list of genes that can affect the immune cells and their response/survival within the tumour microenvironment, this may also include genes that do not have any effect which is also important to define. This will be in the format of publications in peer-reviewed scientific journals as well as presentations at local, national and international scientific meetings. All data will also be released to open data sources. Depending on the availability of reagents we may also identify potential drugs that could go into clinical development.

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

Short term:

Other research scientists will benefit from lists of genes that can affect immune cells and their response to tumours as well as genes that have no effect. They will also benefit from understanding if these genes influence general immune cell biology or act specifically within the tumour microenvironment.

For clinical colleagues the understanding of gene function within the immune system will add further candidate genes and additional information for the identification of causal genes for human immune disorders.

Long term:

Other research scientists will benefit from new understanding for the effect of the tumour microenvironment on immune cell function and the genes which can regulate this.

To the wider pharmaceutical industry and patients this could lead into the development of new anti- cancer therapies.

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

We collaborate with several academic researchers in related fields sharing data, methods and reagents. More broadly through our funding we have strong links to several pharmaceutical companies which allows early triage and feedback on the data and direction to help ensure the most promising targets are followed up. Via open access data release sites all data will be released and if possible negative results will be published to allow others to benefit from the knowledge.

Species and numbers of animals expected to be used

- Mice: 5770

Predicted harms

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

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

We are using mice and typically adult mice because we need a fully developed immune system for our studies which occurs after about 6 weeks of age. The mouse immune system is highly similar to that of humans and allows us to translate our findings. We can also access key immune tissues as the blood only contains certain cell types and the immune response can be restricted to particular areas of the body. There is also a large range of high quality reagents which ensures that the data is reproducible and robust. There are very good models of a variety of cancers for use in mouse and these have been successfully used to identify new treatments which cancer patients are using.

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

We estimate that 55% of mice will be used for the generating mice to be used in our experiments or for the collection of tissues. When they are used in breeding they will be mated with other mice carrying the required modification to their genetic material and we will sometimes have to take a small piece of tissue (normally from the ear) in order to check that the offspring contain this information. For mice used in breeding they will typically be kept up to a year of age. For those mice that are used for tissue collection this may include taking small amounts of blood while they are alive to check the presence of particular cells or after killing to take particular immune organs in order to isolate cells. These cells may be studied in the laboratory and some will be transferred into other mice to use in experiments. The mice used for the collection of tissues are typically aged up to about 6 months although sometimes we may investigate the effect of age by keeping them up to just over 1 year of age.

We expect about 5% of the mice to be given other immune cells by injection into a vein or a body cavity in the abdomen. They will then have small amounts of blood collected so we can track what the cells are doing. In some cases we may give the mice some compounds by injection or in their food or water which we predict will affect the cells in order to determine the role that they have.

About 40% of mice will be administered cancer cells with a small injection to their side or a vein which will either develop as a lump or within the body. Some of these mice will have small amounts of blood collected from peripheral blood vessels while they are awake so we can monitor the response by tracking the number and type of cells present. In some cases we may inject the mice with additional immune cells into a vein or into a body cavity in the abdomen to be able to determine if the alteration we have made makes the cells better at getting into the tumour or can respond better which would make the tumour get smaller. We may also give the mice some treatments similar to those that are used in hospital to discover if what we have identified can help these treatments work better again monitoring the tumour size. These treatments may be administered by injections into a vein or into a body cavity in the abdomen or where possible in the food or water. In some cases we will image the mice by briefly anaesthetising the mice and injecting them if needed (via a vein or into a body cavity in the abdomen) with a chemical that glows in the cells that we have previously given to the mice, this allows us to track where the cells are within the body over time. At the end of the experiments the mice are killed and tissues are collected in order to complete other studies on the material. The mice are typically kept for 2-3 months on these experiments but a small number will be kept for longer (up to 6 months) if we are investigating if the tumour can return or the mice are 'cured'.

Around 2% of mice will be used in the generation or recovery of genetically altered lines where they may have a small surgical procedure performed in order to transplant embryos

or to make them infertile. Alternatively they will be given drugs via an injection into the skin or a body cavity in the abdomen, to increase the numbers of eggs that they produce to allow us to cryopreserve the genetic alteration similar to the process of in vitro fertilisation. This allows us to freeze embryos and sperm for the future and share with other researchers around the world, without needing to constantly breed the animals or transport them to other countries.

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

The genetic alterations that some of the mice in the project carry are not expected to have any effect on the mice and they would live a normal life when housed in our facility. As the study can be affected by the condition of the mice where there is evidence of fighting the mice could be solo-housed or additional enrichment added to the cage in order to reduce the impact.

After administering the cancer cells the mice are typically not affected by the developing lump on their side or tumours within their bodies which normally occurs in the lungs. On rare occasions if the mice have a severe defect with the immune system the cancer cells will grow very fast and the lump can become irritated which can lead to the skin surface breaking and this is a humane end point and the mice would be killed. Alternatively if the mice have cancer cells in their body they could appear to struggle with breathing or have an enlarged body in both of these cases the mice would be killed.

When we administer the treatments the mice do not typically show any side effects, if the response is good they can show a little sickness behaviour similar to a mild cold that normally resolves within a day. Occasionally if the effect is too strong or there is an unexpected interaction with the cancer cells or the immune cells they can develop signs of autoimmune disease which typically presents with diarrhoea and other digestive issues, in this case the mice would be killed.

Sometimes if we give substances to the mice in their food or drinking water this may taste different and so they can avoid drinking/eating. Any mice that are put on a modified diet/water are weighed before starting and at regular intervals to check that there are no problems with weight loss. We can also add some sweetener or other substances to mask the flavour.

Expected severity categories and the 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 62%
Moderate 37%

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

- Killed
- Kept alive
- Used in other projects

Replacement

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

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

The immune system is very complicated as it is spread out across the body with specialised regions which is not possible to model in a dish.

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

We considered a variety of laboratory based models such as cancer cell and immune cell cultures and also tumour organoids.

Why were they not suitable?

The simple co-culture of immune and cancer cells does not allow us to fully model the complexity of the immune system where a key aspect is the specialised microenvironment that exists within a tumour and also the process of migration of various cells around the body. Tumour organoids do have more of a microenvironment, however, they need many inhibitors and growth factors to be maintained which often effects the function of the immune cells. Also it is not possible to address how immune cells can enter or are prevented from entering the tumour microenvironment in a tumour organoid and also address questions regarding the role of other specialised immune sites that are only formed in a living animal with an immune system that is similar to that of humans.

Reduction

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

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

Pilot studies are used to establish the correct dose of reagents/cells, timelines, and to generate baseline data for new experiments. We have refined many of the experimental procedures we are following to ensure that they are robust and reproducible while enabling the detection of effects 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?

All downstream analysis on tissues is performed blinded and where possible mice are randomised to treatment groups across cages/genotypes, this allows for more robust measurements of effects. This together with optimised and refined experimental procedures ensures studies are performed in the most robust and reproducible manner to generate the data with the minimum number of animals. This can be achieved by pooling the data from smaller cohorts over time to reduce batch/cage effects and using specialised statistical methods that enable the analysis of this type of experiment.

Appropriate controls are used (this maybe WT animals or mice injected with control substance/cells).
Online resources such as the NC3R's experimental design assistant and the PREPARE guidelines will be consulted.

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

By standardising the experimental protocols this allows the analysis of data to be achieved overtime and the pooling of data from smaller cohorts. This is especially useful in the case of genetically altered lines where there maybe an effect of survival and/or fertility to allow the breeding to be kept to a minimum level as we do no need such large numbers of breeding mice to generate the number of animals to go into our experiments. Also with standardised experiments and the completion of pilots we are confident that the outcomes are robust and reproducible. Animals that enter experimental procedures are health checked as we have observed that fight wounds greatly impact on the outcomes and these mice are excluded.

Where possible we share tissues with other groups and we can store tissues from animals for future ex vivo studies. We also keep up to date with resources and guidelines such as PREPARE and the NC3Rs experimental design assistant.

Refinement

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

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

Many of the animals used will be genetically altered this could be to add a gene or reporter or to delete a gene or set of genes depending on what is required for the study in question. None of the genetically altered animals have any clinical signs under normal conditions.

For some of the animals we want them to develop tumours which we do by administering cancer cells to their side or in the blood stream, here we follow current national and international guidelines and use the smallest needle and volume to minimise the effect on the animals. The resulting tumours will grow as a small lump on their side or within the lungs and the animals do not typically exhibit any clinical signs. We have monitoring in place to identify animals that are performing in an unexpected manner and for the tumours on the side they are regularly measured and checked to ensure they do not cause any discomfort. There is a maximum size that the tumours can reach before the animal is killed and as the data is routinely reviewed during the experiment if the scientific end point has been achieved we will stop the experiment early.

We also need to administer immune cells or substances that can affect the immune system this is done by the least invasive route possible for the substance and following current

national and international guidelines using the smallest needle and volume to minimise the effect. When more than one substance is to be administered these are combined where possible to reduce the overall number of injections and the site of injection is alternated. We will also administer substances in the diet/drinking water where possible.

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

We considered using lower animals (such as zebrafish) but the translational potential of these in the context of immune-tumour interactions is not yet known and thus mice are considered the gold standard for the development of new therapies in this field. As we are studying the role of the immune system we need to have an intact immune system that is comparable to that of humans and for this reason mice are the best model. We need to allow time for tumours to develop to be able to assess the effect of genes on the ability of the immune system to control the tumours and so these experiments can take weeks or months not enabling the use of terminally anesthetised animals. As the immune system isn't fully formed at birth we need to use adult animals for our experimental work.

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

We have a lot of experience with the proposed models and have refined and developed the clinical monitoring, however, we do always keep up to date with new developments and will look to implement them. Any animals that are to enter an experimental protocol which requires several interventions (such as administration of substances, tumour monitoring etc) will be habituated to handling prior to the experimental procedure starting.

We will constantly research new methods that will result in fewer interventions being applied to the mice or to enable the administration of substances via a less invasive route (such as in the diet/drinking water) and these will be implemented.

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

With regard to preparing our work for publication we follow the ARRIVE and PREPARE guidelines.

We follow the American Association for Accreditation of Laboratory Animal Care and the Laboratory Animal Science Association guidelines for administering substances and withdrawal of blood. For our tumour work we follow the guidelines of the National Cancer Research Institute.

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

We subscribe to the NC3Rs email updates and also receive regular updates from the named people at our establishment. There are also several online sources of advice such as the NC3Rs website and Norecopa.

12. Investigating medicines with unknown mechanisms that target metabolic and immune 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

Diabetes, Cardiovascular disease, Inflammation, Metabolism, Therapy

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

To identify hitherto unknown mechanisms of drugs on metabolism and the immune system.

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

Why is it important to undertake this work?

Often, we do not know how medicines/drugs that were identified a long time ago really work. Examples include metformin (used to treat diabetes), colchicine (for gout) and salicylate (for inflammation). These drugs also exert effects that might be of use in treating other diseases such as cardiovascular disease (metformin, colchicine) or diabetes (salicylate). Unfortunately, because we don’t know enough about how these drugs work,

we can’t develop newer agents based on these properties. These are needed because although effective, these drugs often have challenging side-effects. Our research is aimed towards understanding the basic mechanisms by which medicines of unknown mechanism exert their protective effects.

Mice and humans are largely built from proteins generated from a genetic blueprint encoded by DNA. Most medicines work by changing the function of proteins, or other molecules that are made by certain proteins called enzymes. These proteins are known as drug 'targets'. The main way we study mechanisms of old drugs is to carry out genetic manipulation of suspected targets of the drug in mice. Then we incubate cells taken from the mouse with the drug, or we give the mouse the drug and see if the genetic manipulation has changed the drug action, in a way that can tell us anything about how the drug works.

Through this work, we may develop a better understanding of which targets in cells are the best ones to pursue to design new drugs with properties similar to these older drugs. Such agents could benefit patients who cannot take the older drugs themselves, for example because of challenging side effects. It is also possible that the work could help in 'repurposing' of the original drugs (or new ones based on them) for new patient groups. Repurposing existing medicines can save a lot of time and money because it can be quicker than generating a new medicine from scratch. Trials can be accelerated as, for example, there may be no need for lengthy pre-clinical studies into toxicity and so on because much of this groundwork is already known.

For this project, we are particularly interested in links between the immune system and metabolism. In terms of minimising pain, suffering, distress and lasting harm though, animals will not need to display outward signs of inflammation, nor is it expected that they will become immunocompromised. Similarly with metabolism, the aim is not to cause serious metabolic malfunctioning like diabetic ulcers. We are interested in more subtle effects of the drugs on immune responses and metabolism, particularly as this can correspond to early therapeutic windows in humans, such as type 2 diabetes and Left Ventricular Hypertrophy, the latter of which can be thought of as early stage cardiovascular disease that is often triggered by metabolic dysfunction.

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

In lifetime of project- new information collected into publications and other dissemination

We will have found out new information on how old drugs work and we will have published it. The team has a good track record of publishing data generated. Publishing will be a way for other researchers around the world to build on our research. The work will also have been presented at scientific meetings and at Patient Public Involvement (PPI) sessions.

Long-term (after project):

The research could inform stratification of clinical trials for repurposing. Later it could lead to new medicines, or repurposing of the old drugs as new medicines.

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

Short and medium-term (during and at the end of the project)
We will expect to provide new published data, of benefit to other biomedical researchers in

the field (clinical or non-clinical). The work will be cited and this is one quantitative piece of evidence validating benefit/utility of the information. Others may follow it up with their own research and through PPI activities there will be wider understanding of the utility of the investigation.

Long-term (after project)

The drugs we are studying were identified by accident, so we do not know their targets very well just now. In the longer term the intention is to develop new information that might assist in the development of new medicines based on current effective older medicines, but without their side effects, or with reduced side-effects compared with the original medicine. In practice, the new information might identify a new drug target that new medicines can then also be developed to target.

Alternatively, the new information might allow the existing medicines to gain new uses. For example, the new information might assist in 'stratification' of patient-groups for trials. Stratification is basically a way of focusing a trial for a medicine to a group of patients more likely to benefit. You do not trial the whole patient group, just a subset based on evidence they are most likely to benefit. The older medicines are being trialled for different diseases than they were originally developed for, termed repurposing. Stratification has recently become much more common in cardiovascular drug trials than it used to be. Evidence-based stratification of older medicines in repurposing trials will become much easier if we know more about how they work.

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

The team collaborates widely and has an excellent record of publishing and presenting data at conferences. Publications will be open access and larger datasets will be deposited in relevant public databases. We participate in PPI events, which maximises exposure of the work to non-specialists.

Species and numbers of animals expected to be used

- Mice: 3500

Predicted harms

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

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

The main interest of the team is in reducing diabetes and cardiovascular disease, which is often associated with diabetes. Rodents represent highly useful models of human immune and metabolic dysfunction underlying these diseases. We will mostly use adult mice as these simulate best the age- related immune and metabolic dysfunction that we intend to model.

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

We may use animals where their DNA blueprint has been altered to change the function of something (usually a protein) we are interested in. Typical interventions might include

injections, and/or diet interventions.

Typically, animals will experience mild, transient pain and no lasting harm from administration of substances, usually by injection using standard routes (intravenous, subcutaneous, intraperitoneal). The injections might be single or be carried out daily for a few weeks. Rarely we may use a gavage tube inserted into the mouth to supply substances by the oral route to the stomach for a few weeks. This causes mild discomfort for approximately 30 seconds. Animals will often undergo changes in diet which are not expected to cause distress, but which may be intended to result in early obesity and/or atherosclerosis, or other metabolic dysfunction. Dietary interventions might be carried out for many weeks, as the effect of diet can take some time to build up. Some diets may result in weight loss due to unpalatability although this is not an intended outcome. Animals will be humanely killed should they lose in excess of 15% of their starting body weight.

Animals will experience mild and transient discomfort from blood sampling. To study tissues we will normally use the methods of euthanasia known to cause the least discomfort and distress.

Occasionally, some methods of tissue recovery may be performed under non-recovery anaesthesia where the animals will only be aware of the anaesthetic being administered and no pain.

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

There are unlikely to be significant adverse effects in any of the mice. At various times of life, animals will be killed humanely, and their tissues analysed.

Animals may need to be briefly restrained for injection or oral gavage, but they will be handled gently by skilled animal technicians to minimise any stress. The injections may cause brief discomfort but the drugs themselves shouldn't have any side 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)?

Breeding and maintenance of GA mice 10% mild and 90% sub threshold. Mice: Mild 90% and moderate 10%

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

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.
Why do you need to use animals to achieve the aim of your project?

We replace mouse studies, wherever this is possible, with other methods such as cell

culture, where we can investigate effects of drugs on cells grown in petri dishes. A process known as CRISPR can even allow individual genes to be modified in cells.
In human studies, we can give the medicine and then measure effects of this on inflammation in the blood. Patient databases are also available. In many cases, these databases have genetic information, and they have medicine prescription data, which is beginning to make it possible to study how individuals' genetic blueprint affects their response to medicines.

These other approaches however can only provide part of the information that we need, with systems required to study the complex interactions that can only be achieved in mouse models.

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

Cell studies and studies on human material as described in the previous section.

Why were they not suitable?

Animal studies fill the gap between these other models, where neither cell culture type nor human studies are possible, or they are insufficient because we need to model the role of a gene in the complexity of an organism or physiological system, rather than just a cell type. Genes are genetic blueprints for individual proteins. When studying effects of drugs on metabolism or inflammation for example, it is sometimes important to use tissues obtained from complex animal models where the gene is missing or altered, or to make measurements in things like blood that are affected by changes in multiple organs. Modifying genes can then be a useful way of deducing whether or not the relevant protein is required for the drug to work in the complex system- there is rarely an equivalent approach that can be carried out in humans and 'pharmacological tools', also known as drugs or chemicals, which can be used in humans, animals or on cells with the gene intact, almost always affect more than one target, making interpretation difficult. CRISPR targeting of cell lines is insufficient because the cells fail to model the complexity of the whole organism.

The animal models we will grow are therefore necessary in many such situations, to validate the role of a gene in the complexity of the whole organism, based on inferences that may have arisen from other studies involving human data, cell culture, pharmacology, CRISPR and other 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?

Calculations using typical variations from our own earlier experimentation, or that of others, enables us to estimate minimum numbers of animals to be used, whilst ensuring that the results are statistically significant. Calculations typically show that we need group sizes of 9-15 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. We will need to include controls with appropriate vehicle with the same group size. Sharing of tissues with other groups will reduce the overall numbers of animals used for some experiments. Good training of staff and use of up to date techniques will reduce variability and is another way that we are able to minimise group sizes.

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 to plan our experiments, particularly randomisation and blinding, sample size calculations and appropriate statistical analysis methods. We will make extensive use of factorial design, in particular, which is a very efficient design in gene knockout studies.

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

We will manage the breeding programmes to ensure that they are as efficient as possible in generating animals with the required genetic make-up.

Where they are not covered by legal agreements prohibiting it, we will share frozen resources generated in this project.

We will utilise non-invasive and minimally invasive measurements to maximise data collection from every animal.

Whilst always striving to minimise the severity experienced by individual animals, we will use multiple time points for each one, to maximise data collection from each individual.

Refinement

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

Which animal models and methods 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 rodent models allow us to examine the impact of candidate proteins on glucose homeostasis (sugar handling in the body) and heart/ circulatory disease. In addition, improved technology now allows for us to remove expression of individual proteins in specific organs, times of life, or modify the protein subtly, or even using fluorescent markers to illuminate metabolism and inflammation. These tools allow us to understand in more detail how these two aspects, metabolism, and inflammation, are linked.
The principles we follow in terms of minimising severity are that we intend mice to appear to be of normal welfare, exhibiting mild severity phenotypes such as obesity, diabetes and early stages of atherosclerosis, consistent with early therapeutic windows in humans. We cannot minimise this further, nor choose earlier time-points, because in humans it is generally not considered ethical to treat metabolic disease nor inflammatory diseases

before they happen, by giving medicines to healthy individuals. If we used only completely healthy animals, and/or chose very early time-points, this would not be a very good model for the de facto therapeutic window of early disease that exists in humans. It will not be necessary for animals to display outward signs of inflammatory disease, nor advanced metabolic disease, such as ulcers. No protocols are severe.

In terms of minimising pain, suffering, distress and lasting harm, animals will not need to display outward signs of inflammation, nor is it expected that they will become immunocompromised. We are interested in more subtle effects of the drugs on immune responses, which we will study ex vivo.

Animal suffering will be minimised by ensuring that many animals are only used to extract tissues, all staff are appropriately trained, and that veterinary assistance is sought as soon as possible in the event of animal distress.

We will use enrichment as described in a section below to ensure that, apart from the intended mild phenotypes, animals are maintained in an optimal physiological state.

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

Mice are an appropriate, well-established model for studies of the mammalian immune system. Less sentient animals cannot be seen as an appropriate model, for example for obesity, which is a property of animals with complex reward circuitry in their brains.

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

Animals are stored in dedicated facilities with housing that includes bedding, refuges and allows expression of species-specific behaviours, such as nest building.
Animals are given enrichment objects such as tubes, swings and gnawing sticks.

We will use appropriate anaesthesia and analgesia.

We will utilise non-invasive and minimally invasive measurements to maximise data collection from every animal.

Staff in the lab have developed a refined method of extracting liver cells, a commonly extracted cell type in the laboratory. This method is more reliable than the most commonly used method and which I hope to publish in due course.

All these refinements will minimise distress and maximise the reliability and repeatability of studies.

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, respectively, in preparation and publication of our findings. This will ensure that data from animal experiments is carried out in the best way we can and can be fully utilised.

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

Information is shared via our NIO. Attending 3R related events, such as NC3Rs events and workshops to keep abreast of 3Rs advances and approaches.

13. Investigation of how lung cancers evade the immune system

Project duration

5 years 0 months

Project purpose

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

Key words

Lung cancer, drug resistance, immunotherapy

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?

In this project we aim to understand how lung cancers avoid being detected and eliminated by the body's own immune system, despite showing clear signs of being defective aberrant tissue. By understanding how the tumour hides from the immune system we hope to be able to pinpoint ways in which immune cells could be re-activated to ensure destruction of the cancer, particularly by combining existing therapies with those that stimulate an immune response against the tumour, thereby potentially improving clinical outcomes for lung 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?

About 20% of all cancers are caused by mutations in a group of interconnecting growth regulatory genes called the RAS pathway. There is a pressing need to develop new

effective treatments for cancer patients where this pathway is important, in particular major killers such as lung cancer, colon cancer and pancreatic cancer. Drugs that block the function of RAS proteins have been developed and recently approved for clinical use, but while they have impressive short-term effects in the clinic, patients stop responding to these drugs after a few months due to the development of drug resistance. There is therefore an urgent need to improve outcomes for patients treated with these RAS inhibitory drugs.

Our research is focused on lung tumours caused by mutations in RAS proteins and we have developed a number of new mouse models of this disease which are designed to mimic the complexity of human lung cancer more accurately. These mouse models involve the transplantation of mouse lung cancer cells that have been altered to have mutations in RAS and other genes. We will use these models to address how we can turn short-term tumour regressions induced by the RAS inhibitory drugs into long-term cures. In order to do this, we plan to investigate how best to combine these therapies with others that target the immune system. It is known that many cancers actively evade detection by immune cells by exploiting natural brakes on the immune system, so called “immune checkpoints”. The use of immune checkpoint inhibitors has been clinically beneficial in some cancer types, such as melanoma, kidney cancer and a minority of lung cancers, but most patients do not benefit from these treatments. We wish to investigate the interaction of the immune system with tumour cells as they die in response to RAS targeted therapies.

We plan to explore what parts of the immune system recognise the dying tumour cells and what is preventing the immune system from then fully rejecting the tumour.

We will test whether various types of immunotherapies might be able to work together with RAS targeted therapies to cause complete tumour destruction.

If successful, this work could have a major impact on the way lung cancer is treated in the clinic and could lead to much improved outcomes for these cancer patients, including long term cures.

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

The experimentation described in this programme of work should advance our understanding of the biology of lung cancers and provide new insights into the effectiveness of potential new therapeutic approaches. Specifically, we aim to determine the best way of combining newly approved drug treatments that target RAS oncogene signalling (such as sotorasib) with immunotherapies, both existing (such as pembrolizumab) and novel.

If successful, this work will provide a framework for conducting new clinical trials in lung cancer with combinations of agents targeting RAS oncogene signaling and also targeting the immune suppressive mechanisms that protect the tumours from immune destruction. These will be of direct significance to lung cancer patients with mutations in the KRAS oncogene (the commonest form of RAS), which accounts for some 500,000 deaths each year globally. With this massive disease burden, any improvements in clinical outcome will be very beneficial overall.

Outputs from our programme of work will be in the following forms:

- Presentation at international scientific and clinical meetings.
- Publication in international scientific and clinical journals.

- Discussions with clinicians and pharmaceutical companies with a view to the setting up of clinical trials to determine the efficacy of using combination approaches against the targets identified in this work.

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

Outputs will be used by:
My group to further develop our ideas and improve therapeutic strategies,
by other researchers to pursue their own ideas about combination targeting of proliferative and immune suppressive pathways in cancer,
by the pharmaceutical industry to assess and prioritise the development of new therapeutic agents within the immune oncology area,
by clinicians for the setting up of new clinical trials to determine the efficacy of using these combination approaches in lung cancer
and ultimately, if successful, by lung cancer patients in deciding between treatment options.

Short term benefits:
Improved understanding of the biology of lung cancer, in particular with regard to the interaction of the tumour with the immune system.

Medium term benefits:
Identification of specific vulnerabilities of KRAS oncogene mutant lung caused by the way in which it avoids detection by the immune system.

Long term benefits:
Development of agents targeting novel immune suppressive mechanisms in KRAS oncogene mutant lung cancer (likely in collaboration with others, including industry).
planning and conducting clinical trials with combination targeting of pathways determined in this work (in combination with clinicians and possibly industry).

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

As well as publishing our results in scientific journals and disseminating at scientific and clinical research conferences, we will also engage in public outreach to increase awareness of cancer symptoms and therapeutic options. In addition, we will engage with pharmaceutical companies in order to improve cancer treatment through collaborations. We will publish negative datasets to provide information about unsuccessful approaches.

Species and numbers of animals expected to be used

- Mice: 45,000

Predicted harms

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

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

The area of research covered in this project licence focuses on the development of lung

cancer and the interaction between lung tumours and the immune system. The studies and experiments in this project track living, evolving cancer cells as the disease progresses and changes in response to cancer therapies. This requires the complex interplay between the cancer cells and the immune system, which is made up of dozens of different cell types and therefore cannot be replicated accurately in an in vitro system. As experimental therapies will be employed which have not yet been used in man, we cannot conduct this work on human volunteers, but require an animal system which models the human as closely as possible.

The processes involved in cancer formation and evolution are very similar in the mouse to in man and the function of the immune systems is also highly similar between the two species. The relatively fast reproductive rate, short life span and small size of the mouse makes it a tractable model for experimental use in the laboratory. In addition, it is important that the mouse has been used much the most extensively as a cancer model, so the pathology and genetics of the disease is much better understood in mice than in other model organisms.

Experiments will be carried out principally on adult animals, mimicking the age distribution of lung cancer in humans, which affects almost exclusively adults.

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

We are investigating the development of lung cancer and the interaction between lung tumours and the immune system.

Typically, lung cancer cells will be introduced into immune competent adult mice of the same genetic background to form tumours. This transplantation of cancer cells will typically be carried out by injection into the tail vein of young adult mice, leading to the formation of tumours in the lung, where the cells end up being deposited by the circulatory system. These tumours form in the same location as the tumours from which the cells originated and are likely to model the human disease most closely. Another way of introducing tumours into the lung is by surgery to give a single tumour at a known location. Alternatively, tumour cells can be introduced by subcutaneous injection, which can allow more straightforward measurement of tumour progression.

Once tumours have formed, typically 2 to 4 weeks after injection, mice will be treated with therapeutic regimes, usually involving the introduction of drugs, either orally or by injection, usually with treatment every day. Mice may be culled after 1-2 weeks of treatment in order to recover tumours, which will then be analysed for changes in their cellular constituents, or they will be followed for longer periods of time whilst receiving therapy. During this period the size of the tumours will be measured, either by direct physical measurement of subcutaneous tumours, using callipers, or by in vivo imaging, for example using micro-CT or other non-invasive techniques whilst under anaesthesia. In these longer-term experiments, onset of clinical signs of tumour burden will be evaluated using health status endpoints – in other words, mice will be followed until they show the first signs of deteriorating health, at which point they will be culled. These survival experiments will typically last between 1 and 3 months.

In addition to the formation of tumours by transplantation, we will also use genetically engineered mice which may either spontaneously develop cancer or do so when a gene modifying agent is introduced, for example an adenovirus expressing a recombinase gene, which can be delivered to the lung by inoculation via the mouth into the airway.
We always aim for all our experiments to last the minimum time required to answer the

specific scientific question being asked and aim to use the least invasive methods possible.

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

In most parts of our project where the focus is on lung tumour development and therapy, experimental animals will form tumours, either in the lung or in a subcutaneous location. It is possible that these tumours could cause adverse effects on the animals, although everything possible will be done to mitigate this. In the case of subcutaneous tumours, ulceration may occur as tumours get larger: to avoid this, strict limits are in place regarding their size and any animals showing signs of ulceration will be culled. In the case of tumours in the lung, as the tumours get larger, they may impair lung function and make breathing harder. To avoid this, mice showing signs of breathlessness or excessive weight loss will be culled. Lung tumour size may also be followed by micro-CT scanning and mice culled that show signs of excessive tumour burden. In addition, the therapeutic drug treatments given to the mice could lead to toxic side effects; these will be managed by careful following of the health status of the mice and culling mice that show signs of weight loss or decreased health status. The mice will only experience these clinical symptoms for a very limited period of time as all of these signs are clinical endpoints of the experiments.

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

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

This licence contains multiple protocols where some are of mild (ca 70% of all animals within this licence) and some have moderate severity. For protocols where the aim is to investigate tumour growth, we expect a majority (95% for subcutaneous tumours, 80% for orthotopic tumours) to reach moderate severity due to surgery, repeated procedures or tumour burden. In all cases, we aim to limit the number of animals and the severity of procedures in accordance with the 3R's.

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

- Killed
- 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?

Valuable studies of human cancer can be performed using human tumour material and cultured cells, and both these approaches are being undertaken by my lab. However, mechanistic understanding of cancer pathogenesis and, in particular, the response of tumours to experimental interventions requires the use of living animals. The development and function of the immune system, a particular focus of this work, involves dozens of 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. We are using extensive in vitro tissue culture work and pathology analysis of human clinical samples in addition to these animal experiments, including computational modelling, but the mouse is required for in vivo validation of hypotheses generated from these systems. Other smaller experimental animal models, such as the invertebrate fruit flies and nematode worms, do not have adaptive immune systems and therefore are not suitable for these types of study.

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

We are using extensive in vitro tissue culture work using both mouse and human cell lines derived from cancers. These are studied both in conventional, two-dimensional tissue culture and also in three- dimensional culture, including organoids, which is thought to better mimic the tumour structure. We have followed developments on the use of organ-on-a-chip models and will seek to improve their accuracy for modeling human tumours by incorporating mixed cell systems. We also use heterogenous culture systems with different cell types are represented, such as immune cells, not just the tumour cells. At present, mixed culture systems are limited by the complexity of the tumour microenvironment and also the difficulty of culturing some of the cell types found in tumours. In addition, we carry out pathology analysis of human clinical cancer samples to determine the make up of the tumour microenvironment and the phenotypic state of the cells that constitute it. Furthermore, we have used primary culture of human clinical material from surgical resection of lung cancer under organoid conditions, with the possibility of adding back autologous immune cells cultures derived from peripheral blood samples of the same patients. This may allow study of the interaction of immune cells with tumour cells in vitro in a setting that is at least to some degree relevant to human cancer. However, the availability of material to carry out this type of experimental work is extremely limited.

Why were they not suitable?

Although we have gained useful information from these systems, the tissue culture methodologies cannot fully mimic the complexity of tumour interactions with immune cells and vice versa that are seen in living tissue. The immune system is made up of dozens of major cell types, and hundreds of subtypes and it is impossible to have all of these adequately represented and maintained in any in vitro system. Study of pathology samples can also provide very important information, but it is not possible to intervene to look at the functional effects of targeting specific components. In order to accurately investigate the interaction of tumours with the immune system and the effects of drug treatments on immune mediated responses, studies need to be performed in an in vivo setting as there is currently insufficient knowledge about how to model in vitro these enormously complex processes that involve dozens of cell types, operating in different ways in different locations (tumour, lymph nodes, bone marrow, spleen etc...) within the body. In vitro studies lack the majority of components known to be present in the tumour microenvironment, which include T cells, B cells, macrophages, neutrophils, endothelial cells, fibroblasts and many more cell types. They therefore cannot replace accurate mouse models for the study of how cancer interacts with host tissues and cells, including the immune system. Only whole animal experiments can provide both the complexity of the human clinical situation and the ability to intervene to address functional 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?

We have estimated the numbers of animals used within this licence based on the ongoing projects in the lab, the number of people working within this licence and previous experience of animals required for projects of this scale. The work proposed here is the continuation of projects that have been ongoing in the lab, so we have considerable experience in estimating the animal numbers required to provide robust statistically valid conclusions. 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 have 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 the first place, we have used in vitro cell culture systems to define a limited set of hypotheses that merit testing in animal models. We have also carried out detailed analysis of the relevant scientific literature to ensure that the experiments we undertake are not redundant with those being carried out elsewhere. We then perform an initial, small-scale study to investigate the effect size of the treatment or intervention that we are investigating. Data from previous, similar experiments, by our laboratory and by others, are also used as a guideline. These results are then used to decide the number of animals required for the study using power calculations such as the NC3R's Experimental Design Assistant. We also utilise other aspects of the EDA to build experimental diagrams, receive feedback on experimental design and implement randomisation strategies which can reduce bias, improve the robustness of data and could therefore reduce the numbers of animals used. Trained statisticians are also available if additional help is needed in this regard. Individual experiments involve planning and consultation within our team, colleagues and external collaborators. Experimental strategies, including consideration of the implementation of randomisation and blinding, are analysed before, during and after the experiments, to refine and reduce animal numbers if possible. Experimental bias is reduced by blinding the identities of experimental mice to the research staff who measure outcomes. For each experiment, tumours will be induced in age matched mice, thereby reducing the variations within the experiments and as a result minimize the number of animals needed. Both sexes of mice will be used, unless there are clear scientific reasons not to do so.

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

Mouse colonies will be actively managed to ensure that the basic principles of mouse breeding will be adhered to and only the minimum number of animals required for the experiment are generated. In addition, the use of in vivo imaging methodologies such as micro-CT scanning greatly reduces the number of animals needed compared with end point assays as each mouse can be followed over time and inter-mouse variability is

internally controlled for. We strive to maximise the data generated by each animal, with multiple different experiments using tissues from the same animal. This is done both within the group, and where possible, in collaboration with other research groups.

Where possible, mouse strains are bred to homozygosity in order to reduce the number of animals born with unusable genotypes. In these cases, every animal born for the experiment can be used, which reduces the number of animals required, particularly for complex genotypes, leading to a reduction in animal usage. Cryopreservation of gametes, embryos, tissues and cells is routine at our establishment and will ensure that the minimum number of mice is bred.

All experiments are carried out using inbred mouse strains to limit genetic differences and therefore leading to fewer animals being required to reach a reproducible conclusion. Where feasible, we aim to generate cell lines, histological-tissue samples and other archived resources in anticipation of future analyses.

Refinement

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

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

We will be using two different types of mouse lung cancer models in this project, genetically engineered mouse models and transplantation models of cancer. The genetically engineered mouse models we use generally do not experience any harm prior to tumour induction by the introduction of gene modifying agents. By using intratracheal administration to induce tumour formation, we limit the tumour induction to the lung and reduce risks of systemic effects that potentially could occur. Similarly, the transplantation models limit tumour growth to either the lung or up to two subcutaneous sites.

Strict tumour size limits are in place to ensure mice do not suffer and rigorous health criteria, such as weight loss limits, ensure no animals are left in pain, but are culled at the first sign of distress or ill health. The tumour models chosen in this application reflect both the current clinical and in vitro studies ongoing in the laboratory and with collaborators and will complement both. The methods outlined in the various protocols have been chosen to limit both animal numbers and suffering whilst at the same time providing robust scientific data. Most of the orthotopic transplantation experiments designed to introduce tumour cells to the lungs with the aim of inducing orthotopic growth will involve tail vein injection, but in a smaller number of cases a more time consuming and invasive surgical procedure will be used that allows the establishment of a single tumour, which may more closely mimic the situation in lung cancer patients. Invasive recovery procedures are kept to the minimum required for the experiment; analgesics and anaesthetics will be used where necessary.

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

Less sentient models such as the fruit flies and nematode worms can provide fundamental insights into basic biology, but they are not suitable for understanding the complexity of tumour biology especially in terms of interactions with the immune system. Invertebrates have very different immune systems to humans and mammals in general. By contrast, mouse tumour biology closely models many aspects of human cancers. Extensive published data have shown that mouse models can accurately mirror data from human clinical trials and provide predictive data to improve clinical strategies. Due to the prolonged time frame of cancer development in mice, it is not possible to carry out these experiments in terminally anaesthetised animals. Experiments will be carried out principally on adult animals, rather than neonates, mimicking the age distribution of lung cancer in humans, which affects almost exclusively adults.

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

At our establishment we have access to excellent veterinarians and animal technicians with clear procedures in place in the case of animal concerns, should these arise. We are constantly aiming to improve our experimental models to ensure that we are minimising any harms to the animals. All animals are routinely monitored for adverse health issues. To minimise the harms to the animals, analgesics and anaesthetics will be used where appropriate and we follow local rules and guidance on post-operative care and pain management. We have clear harms criteria for the animal staff, enabling any mice that approach the humane endpoint to be humanely culled as early as possible. We thereby also increase the reproducibility of the experiment, which in turn reduces the number of animals needed to reach solid scientific conclusions. We are evaluating the use of various monitoring methods on animals with lung tumours, to see if tumour burden is detected earlier than by weight loss, potentially allowing termination of the experiment before clinical signs can be observed in the resting animal.

We have chosen administration routes and methods for inducing tumours to minimise the welfare burden for the animals, while modelling the human disease as closely as possible. We use imaging techniques such as micro CT to help with the early identification of internal tumor burdens, allowing for early time points to be utilised and reduced welfare burden for the animals. Animals are monitored very closely by imaging for internal tumour development to ensure that there is the lowest possible welfare burden on the animals and that the development of any potential health problems can be anticipated before they occur.

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

Members of the Joint Working Group on Refinement: D. B. Morton (Chairman), M. Jennings (Secretary), A. Buckwell, R. Ewbank, C. Godfrey, B. Holgate, I. Inglis, R. James, C. Page, I. Sharman,
R. Verschoyle, L.Westall & A. B. Wilson (2001) Refining procedures for the administration of substances https://doi.org/10.1258/0023677011911345
P Workman, EO Aboagye, F Balkwill, A Balmain, G Bruder, DJ Chaplin, JA Double, J Everitt, DAH Farningham, MJ Glennie, LR Kelland, V Robinson, IJ Stratford, GM Tozer, S Watson, SR Wedge, SA Eccles, An ad hoc committee of the National Cancer Research Institute Observers: V Navaratnam17 and S Ryder, (2010) Guidelines for the welfare and use of animals in cancer research https://doi.org/10.1038/sj.bjc.6605642
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https://www.lasa.co.uk/wp- content/uploads/2018/05/Aseptic-Surgery.pdf
Smith, A. J., Clutton, R. E., Lilley, E., Hansen, K., & Brattelid, T. (2018). PREPARE: guidelines for planning animal research and testing. Laboratory animals, 52(2), 135–141. https://doi.org/10.1177/0023677217724823

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

At the establishment where we are located, we regularly receive information from NC3Rs and NORECOPA regarding advances in the 3Rs. New advances are implemented where scientifically possible. When becoming aware of new advances in the 3Rs, we also share these with collaborators outside of our institute. We engage in 3Rs discussions with colleagues/collaborators at lab meetings/departmental meetings/ conferences, attend seminars/events covering 3Rs topics and conduct literature searches for 3Rs advances in our research field. We also use the 3Rs Self- assessment tool for research groups (https://3rsselfassessment.nc3rs.org.uk/) to assess how well our group is engaged with the 3Rs and get feedback and suggestions as to ways to further develop our group's 3Rs culture.

14. Mechanisms of function and dysfunction in electrically excitable cells

Project duration

5 years 0 months

Project purpose

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

Key words

Ion channels, synapses, action potentials, resting membrane potential, plasticity

Animal types	Life stages
Xenopus laevis	juvenile, embryo
Xenopus tropicalis	embryo, juvenile
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?

Our neurons and muscles need to fire electrical impulses and interconnect properly to process our thoughts and actions. This project aims to understand basic mechanisms of how our neurons and muscles wire together and how these electrical impulses and connectivity are affected by 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?

Many diseases affect the brain or its communication with the body when the electrical impulses of our cells or their connections don't work properly. The work under this license

is necessary to understand how diseases such as those caused by neurodevelopmental disorders, genetic mutations or dementia affect the function of cells that fire electrical impulses. Understanding more about how these cells are affected can help us to advance the scientific knowledge for these diseases and develop therapies to eventually prevent or reverse dysfunction.

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

This project aims to investigate how the excitability of cells is affected by diseases caused by alterations in gene expression during development, by mutations in proteins such as ion channels, or by protein dysfunction such as that caused by neurodevelopmental disorders or dementia. Mice and Xenopus tadpoles will be utilised to explore the functional importance of these disease drivers.

The primary outcome of this project will be the publication of research findings related to these topics. The innovative approach taken in this study is expected to yield high-profile publications that will attract attention from both the scientific community and the public. The methods employed will be cutting-edge and may have applications in other research domains.

A potential outcome of this project is to provide information of how particular mutations are affecting the excitability and connectivity of neural or muscle cells.

This work, together with that of our collaborating research teams, may directly inform clinical teams who are working towards diagnosing patients with gene variants of unknown significance. In this way our work will contribute to a better understanding of the disease-causing cellular and systems mechanisms and generate evidence to design future clinical trials. By comparing normal cells and cells with disease-causing mutations, our work may also discover novel molecular mechanisms and help in the generation of new fields of study.

Overall, our research will represent a significant step towards comprehending, predicting, mitigating, and preventing loss of function in major neurological disorders.

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

The work primarily constitutes basic research, with short-term benefits expected in the form of an improved scientific knowledge of biological processes that underpin changes in excitability, neurotransmission and plasticity in neural circuits.

The key beneficiaries of the proposed research are as follows:
Scientific community: The research outcomes will be published in open access scientific journals, reaching the wider academic community and the public. The data and resources will be made available upon publication. The findings will be disseminated through presentations at national and international conferences, and discussions with renowned experts in relevant fields will occur throughout the project. The laboratory-developed techniques will be shared with collaborators and other researchers, and the research will contribute to the training and development of highly skilled neuroscience researchers.

Clinical practice: In the longer term, perhaps within the lifetime of this PPL, we may be able to inform clinicians on the fundamental mechanisms that are affected by mutations in patients. For example, we may be able to determine whether the target system of a mutation is in the brain or the neuromuscular system. This can pave the way for refining

research questions, or inform clinical trial design.

Economic benefit: Our work may pave the way to solidify a diagnosis or drug testing pipeline and may result in spin-out companies or patents.

General public: We will engage in activities to increase public awareness and understanding of science, and the 3Rs in animal research.

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

We will engage with colleagues working in a wide variety of disease models through conferences and seminars. We will share results and approaches prior to publication to enhance our own work and share up to date expertise of our group.

We will engage with patient groups and charity organisations focused on specific genetic disorders to enhance knowledge exchange between communities.

Species and numbers of animals expected to be used

- Mice: 2400
- Xenopus laevis: 500
- Xenopus tropicalis: 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.

In this research project, we will use either Xenopus tadpoles or mice as follows.

Xenopus tadpoles:
Conserved mechanisms of neuronal development, function and plasticity have been discovered in Xenopus embryos and tadpoles and they have been replicated in mammalian neurons. Electrically and sensory-evoked synaptic transmission and plasticity can be analysed in Xenopus tadpoles by assessing identified brain areas. Neuromuscular coupling can also be assessed in explants. Xenopus produces a large number of embryos from a single mating, and similar quantities of synchronized embryos can be generated through in vitro fertilisation. These embryos grow in standardised salt solutions, and multiple animals can be genetically or base-edited in a few hours using CRISPR/Cas targeting. An advantage of using frogs for research is that they lack the brain structures found in mammals that integrate the pain experience or cognitively process distress. Furthermore, pre- metamorphosis tadpoles in which the brain is even less differentiated, allow all the analyses required for this work, to determine the impact of mutations, neuronal or muscular function and sensory integration. The hatchling tadpole brain is composed of a few thousand of neurons (Roberts and Soffe, 2010), compared with the ~10 million present in the adult frog. Comparatively, a mouse brain contains 10 billion neurons and a fruit fly brain contains 100 thousand neurons. This makes tadpoles ethically preferable to mice for certain studies.

Although the vast majority of prior Xenopus studies have been carried out in X. laevis,

these frogs are tetraploid and this had prevented their use to model human genetic disease. However, X. tropicalis, a different species, is diploid (like humans) and possesses a genome highly syntenic to humans. Also, in comparison with an alternative model, zebrafish, X. tropicalis lacks a high proportion of duplicated genes. This simplifies experimental execution of mutation testing and minimises potential confounding results. X. tropicalis has already served to recreate 19 out of 20 potentially disease-causing mutations known as Variants of Uncertain Significance (VUS). The high throughput allowed by X. tropicalis together with prior success in modelling disease is bolstering the confidence in the research community to use X. tropicalis as a species for further VUS-testing and deeper phenotyping. My electrophysiological expertise here will help define whether VUS are causing disease by altering electrical excitability of cells or their neuronal connectivity.

Mice:
The results of studies in mice are highly relevant to studying human disease. Specific brain areas which can be targeted experimentally have conserved functions.

For example, the hippocampus and entorhinal cortex are involved in spatial navigation and memory, and the mouse prefrontal cortex (PFC) is involved in a wide range of executive cognitive functions, including reward evaluation, decision- making, memory extinction, mood, and task switching. Nowadays, the scientific tools available to study mice allow unprecedented access to gene modification or optogenetic activation or tagging of cell-type specific neuronal circuits, which can help us to answer these questions. We will use mice at various stages of their lifespan. We need to use pregnant females to be able to introduce genes in the brains of embryos. These embryos can then be studied at any stage of their life. We will primarily focus on the study of neuronal excitability and synaptic transmission in postnatal and juvenile mice (up to 6 weeks of age); however, some mice will be checked at embryonic stage to assess whether the manipulations have impacted cortical migration or maintained until adulthood to assess the impact of experimental changes in adult mice or test reversibility of disease mechanisms.

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

Xenopus:
Wild type or genetically modified Xenopus embryos will be obtained from approved suppliers and either delivered by approved transport carriers or collected in person and transported following best practice. Tadpoles will be maintained for approximately 2 weeks (not past metamorphosis). Electrophysiological analyses with brain or nerve-muscle explants will be performed ex-vivo.

Mice:
For some of our studies will terminally anesthesise mice and extract the brain for ex-vivo studies. For our other sudies, we will deliver genes of interest in wild type or genetically modified mice in a subset of mice we will also assess how the effects of manipulations are affected by high fat diet or environmental enrichment. The delivery of genes will be done by placing plasmids, viruses or cells within the brain either in living embryos that will be allowed to develop normally, or in juvenile/ adult mice that will be allowed to express the genes of interest for up to 4 weeks. We will then anesthesise the mice and extract the brain for studies of neural function in vitro.

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

Xenopus:

For some tadpoles carrying gene mutations there will be diverse adverse effects including: heart or circulation defects; failure of the brain to develop normally producing seizures or altered behaviour; gut defects limiting the ability to feed properly; and there may even be alterations in overall body structure. These tadpoles will normally be killed humanely after a few days of development when they are approx 15 mm in size.

Mice:
For approximately 40% of mice, we will terminally anaesthetise the animals and collect their brain for in vitro studies.

In other mice, we will perform surgery in mice to deliver genes into neurons.
In utero electroporation involves surgery of the pregnant mother to expose the developing embryos. The embryos brains will be injected with genetic material and a mild electric shock is applied so that the brain cells take up the genetic material. The surgery is completed by suturing the pregnant mother and normal development of the embryos continues. They will be analysed after they are born normally.

Intracranial delivery of DNA, viruses, or cells containing viruses, involves a needle puncture in the soft skull of postnatal mice, or brain surgery in juvenile or adult mice; the skin on top of the head is opened, and a small hole is drilled in the cranium of these mice. Then a very small volume of fluid carrying DNA, viral particles or cells with viral particles (not harmful to humans) is injected into specific brain areas. To incorporate DNA into cells we will use electroporation and a mild electric shock will be applied. In the case of viruses, these will deliver the genes into the mouse neurons, and the cells containing viruses will be incorporated into the brain as part of the brain network.

Good aseptic technique and appropriate use of anaesthesia and analgesia will be used to minimise pain and suffering during and after the surgery. Animals will be monitored carefully after surgical procedures and further painkillers will be administered to reduce suffering if animals show signs of discomfort during recovery.

Animals that do not recover normally will be killed humanely.

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

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

Xenopus:
Abnormalities in development or system function. Moderate (35%) Recordings:
Anaesthesia followed by explant collection is a non-recovery procedure (100%)

Mice:
In utero electroporation will involve moderate severity in the dam (10%) Craniotomy and intracranial delivery will involve moderate severity (10%)
Intraventricular injection of embryos with additional environmental manipulations (environmental enrichment or high fat diet) will involve mild severity (40%)
Anaesthesia and decapitation is a non-recovery procedure (40%)

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

- Killed

Replacement

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

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

To advance our knowledge of how defects on neuronal excitability and synaptic transmission arise, the best available systems are vertebrates. This is because they have similar gene architecture and a highly conserved set of proteins that mediate electrical excitability and synaptic transmission. These vertebrate systems permit experimental readouts from the cellular to the network to the cognitive level, and in this way the mechanisms can be assessed. However, to translate these findings into the clinic it is necessary to design experiments and analyse the data in the context of many other research models. Our ongoing work includes research projects in many of these complementary areas of research either within my own research group or in collaboration. However, investigations in alternative systems alone would not answer the questions proposed in this project license application. Only vertebrate preparation would permit the electrophysiological analyses required for this work to understand the causal links and mechanisms of neuronal dysfunction.

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

IPSCs- Induced pluripotent stem cells Human brain samples.
We also considered the study in invertebrates such as Drosophila and C. elegans.

Why were they not suitable?

IPSCs- Induced pluripotent stem cells. Although these are very accessible for imaging, physiological recording, and biochemical analysis, they have random network connectivity (not anatomically identified circuits). Also, cells are very immature compared to adult human neurons with limited excitability and do not express all proteins of interest, robust synaptic transmission or synaptic plasticity.

Human brain samples. Cortical tissue obtained from neurosurgical procedures contains intact cortical networks and adult neurons which are highly desirable for experiments and to assess mechanisms and pharmacology will be directly relevant to human disease. However, its availability is very limited (approximately one case per month). Also, it is not possible to address communication between different brain areas, and tissue is limited to availability from certain brain areas such as temporal cortex. A combination of research using both human tissue and mouse tissue can overcome these limitations.

We also considered the study in invertebrates such as Drosophila and C. elegans. However, their neurons are extremely small and less accessible to patch clamp electrophysiology. Their synaptic pharmacology is very different from vertebrates, and it would not be possible to model the function and dysfunction of the human proteins we are studying.

Reduction

Explain how the numbers of animals for this project were determined. Describe

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

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

The number of animals used for each experiment has been calculated by considering the known statistical distribution of the outcome measures, the experimental design and statistical tests used to analyse the results, this is based on extensive literature and statistical knowledge and my own prior work.

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

One key step I have taken in my research to reduce the number of animals used in a project is to use experimental methods that allow the measurement of variables in wild type and genetically modified cells within the same animal (e.g. in utero electroporation). This allows the use of paired statistical tests and this significantly enhances the statistical power of my analyses.

Another step I have taken to reduce the number of mice used in my research is to employ a less sentient vertebrate model, Xenopus tadpole, which can be analysed extensively in its larval stage prior to feeding (and prior to protection by ASPA).

However we do require analyses for a short time in its protected, feeding state to maximise the data obtained from this model.

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 computer modelling (e.g. Neuron 8.2 Yale University) to make predictions of neural activity upon changes in excitability or synaptic transmission associated genes.

Xenopus tadpoles:
We will generate and allow development of the minimum number of tadpoles required for this work without excess.

We will fix tissue at the end of electrophysiology experiments to obtain further morphological and gene expression measures from the same animal.

Mice:
We will use slices from the same mouse brain for electrophysiological analyses and immunocytochemistry analyses.

We will perform interim analyses after completion of pilot studies to test whether biologically relevant effect sizes will be observable with a reasonable number of biological repetitions.

We will carefully monitor animal breeding and adjust to provide the required animals for experimental need without excess. Transgenic mouse lines that are not expected to be used in the next year will be archived and embryos will be frozen for future use.
The Project licence holder will ensure that all people working on this project are

appropriately trained and suitably competent to enable a high experimental success rate which will minimise the number of animals used.

Refinement

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

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

Xenopus:
I have selected Xenopus as a research model because I can obtain both single-cell and systems information in vivo from the same animal model. For example, it would be possible to assess the plasticity of central neurons upon stimulation from the optic nerve how this is affected by disease- related genes.

Mice:
I have selected the methods to deliver genes into neurons either by in utero electroporation, in utero viral delivery or following craniotomy in neonate or juvenile/adult mice. This refinement will allow me to express harmful proteins in only a subset of cells (for example Layer II-III cortical neurons in prefrontal cortex or CA1 neurons in dorsal hippocampus) and phenotype single neurons or neural network activity while minimising the systemic effects of expressing such proteins globally in the brain or other tissues that could impact the animal welfare.

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

Here I am using the least sentient vertebrate species and developmental stage I can (Xenopus) for the studies that involve in vivo analyses. I cannot use other invertebrate or vertebrate less sentient animals such as C.elegans, Drosophila or Zebra fish as they don't sufficiently share genomic architecture, neural mechanisms or pharmacological properties with mammalian neurons to model human mutations.
Xenopus work will inform my studies in mice. Mice are required for detailed analyses of synapse composition or analysis of neurons and neural networks. They are the least sentient species with identified brain areas associated with specific cognitive functions.

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

The animal facility staff routinely monitors animal health. Any adverse effects that may develop, particularly in genetically altered strains are followed up with PPL holders and PILs. The animals will be maintained under conditions where their health status can be protected as far as is reasonably practicable. All equipment required for surgery and animal maintenance is routinely serviced.

Surgeries will be carefully managed to minimise harms. Appropriate anaesthesia, analgesia and post- operative care will be implemented with advice from the Named

Veterinary Surgeon:
Xenopus specific: Xenopus tadpoles will be kept in the laboratory in a designated temperature- controlled incubator that will allow daily monitoring daily by PIL holders directly involved in the experimental procedures.

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

I will follow published guidelines issued by Laboratory Animal Science Association (LASA), National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs), and the Royal Society for the Prevention of Cruelty to Animals (RSPCA) to ensure that experiments are conducted in the most refined way.

Xenopus specific: The guidance available is mainly around animal husbandry rather than experimental procedures but I will follow Universities Federation for Animal Welfare (UFAW) handbook The Laboratory Xenopus by Sherril Green and the RSPCA welfare guidelines.

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

All PIL holders and I will access regular training and refresher courses (e.g. PIL refresher every 5 years). The animal facility organises regular user meetings and disseminates a regular newsletter that highlights changes and improvements in animal research including any updates from the Home Office. In January each year, I will conduct annual searches on the FRAME (Fund for the Replacement of Animals in Medical Experiments; frame.org.uk) and NC3Rs (National Centre for the Replacement, Refinement and Reduction of Animals in Research; nc3rs.org.uk) websites to ensure that we can adapt our strategies in line with relevant advances. By staying up to date with the scientific literature in our field of research (e.g. using ncbi.nlm.nih.gov) we will also be made aware of developments that have repercussions on the 3Rs.

Xenopus specific:
My existing collaboration with long-standing members of the Xenopus research community will allow me and my team to attend regular meetings about the use of this model animal, ensuring we know about any advances even prior to publication.

15. Modelling the Prostate Cancer Genome

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

Genes, Genomic instability, Signalling, Cancer

Animal types	Life stages
Mice	adult, embryo

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

This project aims to produce new information about the genes involved in driving aggressive prostate cancer – i.e. which genes do this and how? The long-term goal is to use the data produced in this project to develop new treatments and tests that provide better management of prostate cancer, but the immediate aim of this project is to generate new information.

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

Why is it important to undertake this work?

Prostate cancer is a significant cause of death and morbidity in men. Over recent years, we have made major advances in understanding the changes that occur in the DNA of prostate tumours. These changes turn out to be complex and are likely to change the

behaviour of tumour cells in many ways, determining whether the cancer is stable or aggressive, and influencing the response of the cancer to treatment. Currently, there is limited understanding of why some prostate cancers remain stable for years, but others rapidly progress to a lethal disease that is incurable and difficult to treat.

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

This project will produce new information about the genes involved in driving aggressive prostate cancer – i.e. which genes do this and how?

We expect to identify new opportunities to monitor and treat prostate cancers.

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

Prostate cancer that spreads to other organs (metastasis) is incurable and in the UK approximately 11,000 men will die of this disease each year. Our use of mouse models will help us understand how the genetic landscape of a tumour relates to the risk of developing metastatic tumours. The development of novel model systems will be essential for the development of new treatments to tackle this lethal disease.

In the near term, the findings we generate will benefit the wider clinical research community and our collaborators.

Over the longer term, we hope our findings will benefit cancer patients and their families.

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

Our studies using mouse models are one part of a wider research programme that makes use of biopsy material from cancer patients, research undertaken outside of a living organism (in vitro) and clinical data. This approach provides opportunities for validation of findings made in an animal model and increases the relevance to human disease.

Our findings will be made available to other scientists through collaborations, publication in high profile, peer-reviewed journals and presentations at scientific conferences and meetings. Our Establishment has a policy of ensuring that all publications generated are available on open access to all. In addition, our work has direct translational and clinical applications that we will investigate through collaborations with clinicians at the Establishment and other medical cancer centres worldwide. Data will also be shared with the general public through outreach activities within the local community, social media and other public engagement activities.

Species and numbers of animals expected to be used

- Mice: 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.

This project utilizes mice, which are the least sentient species available with a complex urogenital system that includes hormone responsive tissues and organs such as the prostate. In addition, mice also possess an immune system which can be important for cancer development and treatment. Only adult mice will be used in experimental procedures, however for one of the protocols, embryonic tissues are required to provide conditions essential for prostate tumour growth.

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

Mice will be used to host the growth of human prostate cells which will form either benign tissues or tumours. There are three sites of implantation, and these are specific to each protocol. In one protocol cells are injected into the kidney capsule to provide an environment suitable for the growth of tumours and benign prostate lesions. In a second protocol, prostate-derived cells will be implanted onto the back of the mouse, under the skin, for ease of monitoring as a xenograft. In the third protocol, cells are injected into the tail vein to assess their metastatic potential (ability to spread and grow in non-prostate organs). Tumour growth is not associated with pain during the period in which we conduct our observations. Tumour growth will be monitored regularly by either use of callipers, or by imaging method, following dye injection, for internal tumours. For some procedures that involve surgery, we will administer pain killers and monitor mice closely. The mice will also have blood samples taken either from the tail vein or by sampling from a heart chamber under non-recovery anaesthesia. Occasionally mice may be administered organ preservative whilst under non-recovery anaesthesia to allow us to undertake microscope investigations on slices of selected organs. At the end of a procedure mice will be humanely killed.

In a fourth protocol pregnant mice will be humanely killed, the embryos removed and humanely killed so that embryonic stem cells can be used to promote growth of very slow growing prostate cells.

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

Mice may experience some discomfort as a result of injection or small surgical procedures. We will administer anaesthetics or analgesics as appropriate to minimise these effects. Some mice may experience discomfort associated with growth of sub-cutaneous tumours where scabbing or irritation occurs. Mice will be closely monitored for these effects and will be humanely killed if these symptoms cannot be alleviated within 24 hours.

Mice may also experience discomfort associated with the growth of internal tumours. We will select a humane endpoint for tumour growth assays and closely monitor the mice throughout.

Cancer therapies can cause side-effects due to toxicity. We will administer doses that are known to be well tolerated by mice. If this information is not available, we will undertake pilot studies to ensure we are using an appropriate dose going forward.

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

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

The vast majority of mice are only expected to experience mild to moderate clinical symptoms due to tumour growth before they are humanely killed. We have extensive experience of cancer models in our centre and on our previous licence. Some mice will experience the discomfort of injection of therapeutic agents and labelling agents or oral delivery. We will aim to utilise the least stressful route of administration wherever possible.

Mice that undergo surgery will be anaesthetised for the operation and receive pain relief peri- operatively until pain subsides. Some mice will also have repeated anaesthesia for the purposes of imaging the internal tumours. Whilst loss of consciousness may be distressing this is not painful.

We anticipate that the mice used in this project will suffer either mild or moderate symptoms. Here, we provide an estimate of the proportion of mice likely to experience these symptoms given their use in a particular protocol.

We have taken into consideration the likely impact of each protocol on the mice:
Protocol 1 involves a surgical procedure and implantation of cells in to the kidney capsule. We suggest all mice undergoing this procedure will experience moderate harms, irrespective of tumour growth.

Protocol 2 involves subcutaneous injection of cells. We suggest that if no tumour growth occurs, or tumours remain small, mice will experience mild symptoms. On the other hand, significant tumour growth has the potential to cause moderate harms.

Protocol 3 involves tail-vein injection of cells with seeding primarily into the lung epithelia. Here, even the growth of small tumours has the potential to cause moderate harms, so we place all mice undergoing this procedure in the moderate category.

Protocol 4 is a non-recovery procedure.

The number of mice estimated to fall in each category is tabulated below:

Protocol	Number of Mice per protocol	Mild harms	Moderate harms	Non-recovery
1	200		200	
2	200	100	100	
3	200		200	
4	100			100

The total number of mice used overall will be 700. Of these, we estimate 14% (100 mice) will experience mild effects, 72% (500 mice) will experience moderate effects and 14% (100 mice) will undergo a non-recovery 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?

For the vast majority of experiments we don't use mice, but instead cells grown in the laboratory (tissue culture) and their use enables us to answer many questions regarding specific scientific problems. As this project concerns the genetic changes that occur in human prostate tumours, we engineer human cell lines isolated from surgical specimens to mimic some of the genetic changes found in humans. The mouse studies proposed herein allow us to characterise these cells in ways that cannot be accurately modelled in vitro. During this project we will test:

whether specific genetic changes can drive the formation of tumours,
whether these tumours retain a hormone dependence for growth,
whether these tumours can spread to other organs (metastasis),
whether the tumours 'evolve' as they growth due to genome instability,
whether the tumours develop regions of low oxygen content (hypoxia).

Furthermore, we cannot at this stage model in vitro the changes in hormone signalling that occur during mammalian development, or the interactions the tumour has with its neighbouring cells and the immune system.

NB The approach taken here, ie genetic modification of human cells is intended to replace the need for transgenic mouse strains, reducing our requirement to breed new mouse strains, and therefore represents a considerable 3Rs advance compared to our previous licence.

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

Use of mice will be minimised, where possible, by using in vitro methods , with tissue taken from mice and tested in vitro (ex vivo) and computer-based modelling (in silico) systems. Our expertise in the field of prostate cancer, together with our global network of expert colleagues will alert us to any advancements in non-animal approaches that arise. Moreover, we will continue to assess new ex vivo model systems to compliment the in vitro models. We will continue to test and develop these in vitro and ex vivo systems over the next few years to address how well they can effectively model immune responses to cancer.

Generation of manufactured organs (organoids) or explants will be considered and used as an alternative approach to assess responses where feasible. This would mean that a single tumour (from a single animal) could be used to investigate a number of therapies and reduce the number of mice involved in the study.

Why were they not suitable?

It is important to note that, unlike most other tumour types, prostate cancer cells are not amenable to in vitro culture. Direct culture of localized prostate tumour cells either in conventional or organoid systems is highly challenging. We have worked hard on developing novel models based on non-tumour prostate epithelial cells, and we will continue to conduct extensive work in vitro using these systems, including the introduction of tumour promoting (oncogenic) mutations to these cells. Occasionally, it will be necessary to test the tumour-forming capacity of these cellular 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 will use the smallest number of mice necessary to answer a specific scientific question. Based on bio-statistical advice a minimum sample size of consists of 7 animals per group would be needed. We will recruit 8 animals per group in this study to allow contingency. This allows for detection of effect sizes greater than 30% when comparing control and experimental groups. More subtle effects would require larger groups, but we would be reluctant to use large numbers of mice to prove a subtle effect. This will be backed up by many years of experience in choosing the right number of mice and by seeking further advice on statistical methods, when necessary, to ensure the experiment will be designed properly. We will design very simple experiments in which a group of control mice is compared to a test group. For example, a group of normal mice will be compared to a genetically altered group, or a placebo / standard of care group will be compared to a treated group of mice. This design will allow us to detect an effect size of 30% or greater.

Therefore we think that this simple design will suit our work and allow us to conclusively measure important changes in biology.

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

Our approach is to set the group size before conducting the experiment based on discussion with a bio-statistician or performing Power Calculations based on variation of measurement of the primary endpoint being measured. Eight mice per group is sufficient to allow a valid study design where variability of the primary endpoint is 20%. We accept that this will allow us to only detect effect sizes of 30% or greater when comparing two groups of animals with 95% confidence that the difference is not due to chance. We will only increase the group size beyond eight where either the primary endpoint variation is higher or an effect size of lower than 30% is required.

We also realise it would be very difficult to replicate the huge complexity of the prostate cancer genome using a suite of transgenic mouse strains. Therefore we are seeking to use an alternative approach of implantation of prostate cells in this current application. We expect this to reduce the number of mice to be used in any breeding programmes to support 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?

We will take steps to maximise the amount of useful information we generate from each mouse in order to keep the number of mice used to a minimum. We will carefully preserve with chemicals (fix) and archive implanted tissues so they are available for use in the future.

We will perform as much preliminary work as possible in culture models in vitro and in silico analysis prior to engaging in in vivo studies. In cases where we are testing tumourigenicity of our models, we will first conduct experimental work in vitro (eg migration assays, anchorage-independent growth) to provide evidence that the in vivo work is likely to produce meaningful results, so that mice are not used unnecessarily.

We will use inbred strains and by housing them under identical conditions to limit variability.

We will perform pilot studies using small numbers of mice when information is lacking in the literature/from collaborators, so that the number of mice utilised in experiments is reduced to minimal levels.

Refinement

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

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

Wherever possible we will use experiments involving implantation of human cells with defined genetics, rather than breeding transgenic mouse models of prostate cancer. This ensures that possible suffering associated with development of cancers intrinsic to the host animal is avoided or kept to a minimum. After surgical procedures we will monitor mice for signs of pain and administer effective pain relief for as long as it is required.

Previous experience will be used to guide experiments to help to minimize potential suffering. For any new procedure, we will seek expert advice and follow the most refined techniques available.

For any procedure we will follow the route that causes the minimal burden on the animal's well- being to test scientifically the desired measurable outcome. For example, we are moving away from intra-peritoneal injection of chemical labels to measure cell growth rates to administration in drinking water.
Tissue specific and inducible gene alteration will reduce the potential burden of genetic loss in the whole organism.

We will follow guidelines of good practice administration of substances will be undertaken using a combination of volumes, routes and frequencies that themselves will result in no more than transient discomfort and no lasting harm.

All surgery will be done aseptically (e.g. LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery http://www.lasa.co.uk/wp-content/uploads/2017/04/Aseptic-surgery- final.pdf).

Peri-operative analgesia (e.g. Carprofen administered orally or sub-cutaneously) will be

given and maintained after surgery for as long as is necessary to alleviate pain as measured by the grimace scale and other indicators of well-being.

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

Although genetics can be employed in less sentient species, (e.g. fish), these do not have a prostate, and are not suitable for our work. In order to study prostate cancer we need to use a mammalian species, such as mice, which possess urogenital organs that are similar to the human system, together with the hormonal regulation (e.g. testosterone) of these tissues. Mice represent the least sentient mammalian species in which we can conduct genetic experiments, allowing us to model the genetic changes found in human cancers.

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

Animals will be group housed wherever possible, provided with enrichment and handled by either tunnel or cup-handling. Animal suffering will be minimised by making every effort to keep the tumour models employed at the subclinical levels. Wherever possible, this will be achieved by using non- invasive imaging methods to monitor tumour growth and the development of metastatic disease. In addition, steps will be taken to minimise the severity of the procedures. We have replaced surgical castration as a means of androgen blockade with administration of a chemical (Degarelix) as a non- surgical approach. Finally, we will ensure that all mice receive the highest standard of care, and preventative medicine (including anaesthesia and analgaesia where required) will be used.

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

Surgical procedures will be carried according to the LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery.

Relevant published literature will be used as template for experimental design and decision making (Workman et al., 2010. Guidelines for the welfare and use of animals in cancer research. BJC, 102, 1555-1577).

We will follow guidelines of good practice [Morton et al., Lab Animals, 35(1): 1-41 (2001); Workman P, el al. British Journal of Cancer, 102:1555-77 (2010)].

We will consult the NC3Rs guidelines and monitor refinement where such practices are published (NC3Rs website and elsewhere).

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

By reading 3Rs literature and participating in 3Rs workshops locally and nationally, and through discussing refinements with our animal care staff and vet. I will also read the NIO reports that are circulated within the Establishment. I am a regular attendee and contributor to our Retrospective Review and Licensees meetings and the 3Rs Poster session, all of which take place annually at our Establishment.

16. Origins and mechanisms of allergic and fibrotic lung disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Chronic respiratory diseases, Asthma, COPD and fibrotic lung disease, Airway inflammation and remodelling and lung fibrosis, Prevention, New treatments

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

To assess the mechanisms of acute/chronic inflammatory and fibrotic lung diseases by using novel therapeutic interventions.

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

Why is it important to undertake this work?

Chronic respiratory diseases (CRDs) such as asthma, chronic obstructive pulmonary disease (COPD), and fibrotic lung disease affect a large number of people globally, with

over 300 million individuals suffering from them. They can lead to significant health problems and even early death. Therefore, it is crucial to conduct research to better understand mechanisms behind these diseases for the development of new treatments.

Asthma and COPD get treated with anti-inflammatory steroids, special antibodies (like Anti-IgE, Anti- IL5, Anti-IL5R, Antil-IL4Ra, Anti-TSLP), and drugs that relieve symptoms, such as beta-agonists and muscarinic antagonists (bronchodilator agents).

For fibrotic lung diseases like idiopathic pulmonary fibrosis (IPF), small molecule anti-fibrotic drugs like pirfenidone (p38 MAP kinase inhibitor) or nintedanib (tyrosine kinase inhibitor) are used, but they come with significant side effects.

Unfortunately, there are currently no disease-modifying therapies that can prevent or reverse remodelling and scarring of the airways in asthma, COPD and other CRDs, which remains an unmet medical need.

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

By the time this project concludes, we anticipate achieving the following results:
Our findings will be published in journals where experts review and approve the work.

We'll share our discoveries at meetings where scientists from around the world meet and discuss.

New exclusive rights will be established for inventions we create (e.g. patents)
Promising substances could move forward to their first tests on humans, a significant step.

We might also uncover new specific biomarkers, signs that can tell us about diseases and how treatments are working, helping us better understand health conditions to advance scientific knowledge.

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

In short-term our improved understanding of the molecular mechanisms of airway remodelling, fibrosis and bronchial hyper-responsiveness (BHR) and the potential impact(s) of environmental and exogenous factors will benefit other researchers in the field of lung research.

In mid- to long-term by targeting airway remodelling, fibrosis and BHR using novel compounds will not only improve our scientific understanding of lung disease but has the potential to benefit our patients with asthma, chronic obstructive airways disease (COPD) and fibrotic lung disease as a new and much needed disease modifying therapy.

Looking ahead, mid- to long-term we will seek to forming agreements with big pharmaceutical companies to license our discoveries or creating a separate company based on our research within the university.

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

We are committed to making the most of our research findings.
We will share our work by publishing it in journals that anyone can access, as UKRI requires. We will also talk about our findings at important meetings of scientists from all over the world.

Additionally, we'll provide valuable tools and materials to fellow researchers at our institution or even at research centres worldwide. This might include things like data, animals for study, or tissue and biologic fluid samples.

Our goal is to contribute and collaborate for the benefit of science and society.

Species and numbers of animals expected to be used

- Mice: 4000

Predicted harms

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

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

Mice are often picked as the top choice for studying human lung diseases, and there is a good reason for it: Mice are quite similar to humans in how their lungs work, and they even get many of the same diseases because of similar genes.

For our experiments, we will use wild-type mice and mice that have been genetically changed (for example, mice with a special human gene inserted or mice with the gene turned off). These different types of mice will help us learn more about how genes linked to lung diseases like asthma, COPD, and fibrotic lung disease play a role in causing these lung problems.

We will use mice at different stages of life, from when they are newborns to when they are adults. We know that respiratory diseases can start early in life, even before breathing begins. By studying both young and adult mice, we want to find out if we can do things to change how these disease-related genes work in different types of mouse models. This might help us develop ways to prevent these diseases early in life or treat them later on.

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

Taking care of mice for research involves providing them with a comfortable and controlled environment. Mice are kept in groups in special cages that are kept clean, safe and enriched with toys and borrowing material at a constant temperature. They have freely access to food and water, and their living conditions are carefully monitored to make sure they stay healthy. Researchers keep a close eye on their behaviour and well-being, making sure they're not stressed or in pain. This careful care helps ensure that the results of the research are accurate and reliable.

To study the mechanisms of allergic airway disease with inflammation and remodelling and lung fibrosis we will expose mice to different environmental and exogenous agents (allergens, pro- inflammatory agents and pro-fibrotic agents) at different concentrations and routes (via the nose, via the back of the mouth, into the peritoneal cavity, through a small tube inserted into the main airway) without or with putting the mice to sleep for a short time (anaesthesia) 1 to 5 times per week from 1 week for up to 12 weeks.

To study the therapies to prevent or treat allergic airway inflammation, airway remodelling

and lung fibrosis we will administer small molecules (oligonucleotides and their non-targeting controls (NTCs)) or approved and commonly used drugs in humans (e.g. glucocorticosteroids or anti-fibrotic compounds) at different concentrations and routes (under the skin, into the peritoneal cavity, via the nose, via the back of the mouth, through a small tube inserted into the main airway or through exposure to fine mist in the air (aerosol)) without or with putting the mice to sleep for a short time (anaesthesia) 1 to 5 times per week from 1 week for up to 12 weeks. Sometimes, we will take a small blood sample from their tail vein.

And in many cases, we will gently put a small tube into their main airway through a small surgical procedure after the mice have been made fully and deeply asleep (general anaesthesia) to measure how well their lungs work (lung function test). After this we will take out some fluid and tissues from their body while they are fully asleep and then we will kill them in a special way that does not hurt them without the mice waking up.

For mice that are not having their lung function checked, we will kill in a special way that does not hurt them without the mice waking up.

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

We will give the animals certain amounts of substances in ways that won't cause any long-lasting discomfort or harm.

When we make the animals breathe in agents like allergens or inflammatory substances to study allergic airway disease, we expect them to have a little trouble breathing and maybe breathe a bit faster right after. But this should often get better or may persist in a very mild form.

In the lung fibrosis model, where we are studying scarring in the lungs using a special substance, we think the animals might breathe a bit harder and lose some weight. We will be checking their weight to make sure they're doing okay.

Expected severity categories and the 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, 80% 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?

- Because of ethical concerns, it is difficult or sometimes not possible to directly study how environmental factors affect humans
- To test that modifying certain genes could be useful for treating lung diseases, we must first see their role in the development of lung diseases in animal models.
- Before we can use new treatments on people, we need to test them on animals in real-life situations to make sure they are safe and effective. This helps us figure out if these treatments could eventually be used for humans.

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

We have created specific cell culture systems where we can grow and study lung cells from mice and humans outside of the body. This helps us test new therapies that might help with lung diseases.

We use these systems to look at cells from human lungs and from humanised mice. We are particularly interested in testing in cells pharmaceutics of the class called anti-sense oligonucleotides (ASOs) to see how well they may work against certain lung diseases in the future. We are going to use the cell culture system to test different concentrations of ASOs work the best in these cells and will inform the concentrations use in our mouse models.

Additionally, we are using our experience from studying lung slices in a special way (precision cut lung slices (PCLSs)) to do similar tests on human lung tissues that were removed during lung surgery. We want to find the best ASOs to use, and we're doing these tests alongside other experiments we're doing in living animals to make sure we are on the right track to find new treatments for lung diseases with the least or no side effects.

Why were they not suitable?

The way we study cells and lung slices in a dish or outside the body has its limitations. We cannot use these methods to see how our main therapeutic agents work in models of allergic inflammation and associated lung changes including lung scarring. Such processes are orchestrated by an interaction of different cell types (e.g. structural airway cells and cells of inflammation) in response to different environmental or exogenous factors in a three-dimensional lung structure over time.

For that, we need to use the whole animal in our experiments. This will help us not just to look at how our treatments affect lung function, but also see if they cause any changes in how the airways and lungs are put together in their structure. We will also check if there are any unintended effects from the substances we're 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?

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 12 to achieve the quality of results we need.

We've used our annual return of procedures data and experimental designs 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?

We have peviously employed and will employ the NC3Rs' experimental design guidance and experimental design assistant (EDA) to plan our experimental design, practical steps and statistical analysis utilising the advice and support for randomisation and blinding, sample size calculations and appropriate statistical analysis methods. We will use the EDA diagram and report outputs to support experimental planning with animal users.

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

We will mate male and female mice that have a specific human gene on both chromosomes to create more mice that also have that gene and both chromosomes, and we can use them (male and female) for our tests.

We will also mate mice that have a certain gene missing, but not completely, to make sure we have a mix of mice with and without the gene. This helps us have different types of mice to compare (experimental and control mice) in our experiments.

We will do some smaller pilot tests first to figure out the best timing and amounts of our therapeutic compounds to use for our main tests with the lead compounds we are studying.

After we finish our experiments, we will collect as many body tissues as we can from the mice after they are killed. If we don't need to examine the body tissues right away, we will freeze them so that other scientists who are studying similar things can use them 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.

For our experiments, we will use wild-type control mice and mice that have been genetically changed (for example, mice with a special human gene inserted or mice with the gene turned off). All these mice develop and breed completely normal.

To study the mechanisms of allergic airway disease with inflammation and remodelling and lung fibrosis we will perform pilot studies and will first expose mice to different environmental or exogenous agents via different routes (e.g. through themouth or nose into the airways, into the abdominal cavity, ...) at different increasing concentrations to find the lowest dose of environmental or exogenous agents that starts inducing mild allergic airway disease or lung fibrosis that we can use as disease models in our studies using new substance to treat these diseases.

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 lung structure and immune cell or their lung structure and immune system is too different from the human immune system to provide relevant results.

Most of our mouse studies will be in adult animals as chronic lung disorders such as asthma and lung fibrosis mostly manifest themselves in adult life. Some mouse studies of allergic airway inflammation and remodelling can be performed in young mice as e.g. asthma has often its origin in early life.

All lung function testing will be performed in mice that are fully anaesthetised before they are killed (terminally anaesthetised mice) at the end of the experiment.

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

All animals will be observed up to twice daily for the first 24 hours after challenges with aeroallergens or inflammatory agents or pro-fibrotic agents for the first time and then as appropriate.

Animals treated with pro-fibrotic agents will be followed by regular monitoring of body weight (BW).

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

We will use published guidelines to assist with planning our animal research and testing such as Planning Research and Experimental Procedures on Animals: Recommendations for Excellence (PREPARE) guidelines (Laboratory Animals 2017). We will use further information from the Norway's National Consensus Platform for the advancement of "the 3 Rs" (Replacement, Reduction, Refinement) in connection with animal experiments (www.norecopa.no).

We will also use other available resources such as guidance and publications from the NC3Rs and Laboratory Animal Science Association in the UK.

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

We will regularly check information on the NC3Rs website, we have signed up to the

NC3Rs newsletter and attend Regional 3Rs symposia organised by NC3Rs or UKRI (MRC).

We will also use information from other networks such as Norecopa the Norway's National Consensus Platform for the advancement of "the 3 Rs" (Replacement, Reduction, Refinement) in connection with animal experiments. Additionally we will search the scientific literature for novel approaches for the 3 Rs.

17. Parasitic helminth infections: mechanisms of immunity and immunoregulation

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Immune response, immune regulation, parasitic helminth, vaccination, immune mechanisms

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

To better understand how the genes and cells that are activated following infection with parasitic worms that live in the intestine (intestinal nematode parasites), or following vaccination with molecules derived from these worms, regulate or promote immunity.

To develop and define ways to modulate infection and/or damage caused by gut dwelling worms by exploring ways to alter the 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?

Gut dwelling parasitic worms are extraordinarily common and cause ill health in around one quarter of the world’s population. The project is important for both humans and animals and aims to understand why some individuals are more likely to become infected with worms than others. Developing treatments for parasites is difficult, due to a poorly defined understanding of how the immune system responds to infection and poorly defined

mechanisms of worm infection, development and avoidance of immunity. The project will generate a deeper understanding of how the immune system works following infection by worm parasites. Relatively little is known as to the ways in which the body protects itself following this kind of infection. In the long term, understanding better how the immune system responds or does not respond to parasites will be key to the development of for example vaccines. The ultimate goal is to develop new and better ways to control these sorts of infections.

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

The primary output of this project is to identify the ways by which parasitic worms and/or vaccination with parasite molecules regulate the immune response and the damage associated with infection.

We intend to share our findings with the scientific community and will publish at least three new papers describing novel ways by which gut dwelling worms regulate the immune response or mechanisms by which the host protects itself from infection at the level of cells and molecules. We expect to present our work, in the form of a poster or a talk, at both national and international conferences. In addition, we will continue to present our work to the public within public engagement events and science festivals.

We will also work closely with the Establishment media office to communicate our research to the general public via press releases as appropriate.

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

Medical and Veterinary benefits: Long-term (chronic) disease is a major global problem. In developing countries chronic infectious disease, particularly caused by parasitic worms has a major effect on health on humans. In domestic animals, infection by gut worms also has a major direct health and economic impact worldwide. Our research focusses mainly on the gut whipworm parasite, Trichuris.
However we have included other worms too, as whipworm is one of several parasitic worms that collectively inflict a bigger global disease burden than HIV, malaria or tuberculosis. Through the identification of the key events and processes that occur during the immune response to gut worms we will inform programmes in the field which strive to control infections and reduce the tissue damage caused by infection.

Product outputs: We will gain insights into the immune response to infection with gut parasites by defining the mechanisms involved in resistance and susceptibility to infection. Our new discoveries will enable us to develop experimental vaccines. When a decision is made to place the data in the public domain, we will do this in the following ways: (i) publication in peer-reviewed journals in the fields of infectious diseases (ii) presentations at national and international conferences (iii) delivery of public engagement events (iv) press releases.

Who will use the outputs and how: The research involves basic bioscience and preclinical mouse models. Our work will be of interest to other academics working in the field of immunity to infection with different types of pathogens, and to immunologists interested in immune response in the gut. Our findings will also be of interest to clinicians and the veterinary field.

Time scales: we would expect to continue publishing our work in this area annually as part of an ongoing research programme. Equally we would expect to discuss our new

unpublished data at national/international conferences annually. It is highly likely that we will have identified up to five new drivers of resistance and susceptibility within a 5 year timeframe. It is highly likely that we will have identified up to three new lead antigens and/or antigenic epitopes for inclusion in experimental vaccines within 5-10 years.

Short term benefits: We expect to generate a comprehensive mechanistic analysis of the immune response to intestinal parasitic worm infection defining key genes, cells and molecules involved in acute and chronic intestinal parasite infection. This will have direct application to our fundamental understanding of immunity to gut parasites and also to our broader understanding of intestinal immunity.

Medium term benefits: We expect our fundamental mechanistic work to allow us to develop our work in the area of experimental vaccination and explore novel immunomodulatory approaches based upon the use of parasite derived molecules. We expect other academic researchers to apply the new knowledge we generate to their own experimental systems to test any new paradigms.

Long term benefits: In the longer term we anticipate that our data will translate to studies on human whipworm and other gastrointestinal roundworm infections of man and animals. Our studies in the long term will inform and advance future control programmes. We thus expect the results of our research to play important roles in the reduction of human and animal parasitic diseases.

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

We will submit our research manuscripts to open access journals and we will deposit manuscripts in BioRxiv, the open access preprint repository for the biological sciences.

Sharing our data sets, where appropriate, at conferences and invited talks will allow researchers to test our findings against other types of parasite infections.

In addition to publishing positive outcomes of our research we also strive to publish data where for example a particular gene deletion has no effect on the outcome of infection as this knowledge is useful.

We also strive to share our methodologies and workflows allowing others to adopt our working patterns if appropriate.

Species and numbers of animals expected to be used.

- Mice: 17 500

Predicted harms

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

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

Our studies focus on the infection of adult laboratory mice with male and female gut dwelling parasitic worms, primarily the parasitic whipworm Trichuris muris (*T. muris*). *T. muris* is the equivalent mouse parasite of *Trichuris trichiura* (*T. trichiura*), the whipworm

which infects humans. *T. trichiura* and *T. muris* are virtually identical and therefore the mouse model enables us to develop new therapies to treat *T. trichiura* in humans by understanding better how the infected host responds to the worm, what is needed by the host to get rid of the worm and how the worm tries to dampen down the immune response. We will also infect mice with the rodent hookworm Heligmosomoides polygyrus to model human hookworm infection and *Trichinella spiralis* which can infect humans as well as mice.

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

Mice will receive a parasitic infection. Parasites will be given via the mouth usually on one occasion. Subsequently, mice will be treated with for example with an antibody that will block a key component of the immune system via an intraperitoneal injection (typically 1-2 times a week for three weeks; up to 10ml/kg), and the infection allowed to progress for 35 days when we would normally expect adult stages of the parasite to have developed. During these 35 days some mice will have small volumes of blood withdrawn for their tail veins, with no more than two samples taken in any one week and never exceeding the published guidelines on blood sampling. At day 35 mice will be culled by a Schedule one method and their immune responses analysed.

In other experiments mice will receive one subcutaneous vaccination (up to 5ml/kg) with a parasite derived molecule or molecules in a modern adjuvant for example alum or a virus like particle. Mice will receive a second subcutaneous vaccination for example 10-14 days later prior to infecting orally with a gut dwelling parasite (for example *T. muris*). The infection will be allowed to progress for 35 days when we would normally expect to see adult stage parasites and then the mice will be culled by a Schedule one method to see if the vaccine has protected the mice from the infection.

The collection of bacteria in the gut (the microbiome) has a well-established ability to modify immune responses. In experiments involving “faecal transplants” we will deliver a faecal “slurry” orally to recipient mice to see if we can transfer the immune response seen in the donor mice to the recipient simply by transferring the faeces. Oral delivery (via the mouth - “oral gavage”) is a well-established method for transferring microbiomes between mice and is a mild procedure in itself, causing no more than transient mild stress at the time of delivery.
In some experiments we will need to identify which compartment of the immune system is responsible for, for example, resistance to infection. This can be done by irradiating mice to remove one of the two compartments (the immune cells, leaving the non-immune cells) and then restoring the immune cell compartment via a specific type of bone marrow transfer creating the “bone marrow chimaera”. These mice, once the bone marrow has established, may then go on to have a vaccination and an infection in order to identify which type of cell is important in protection.

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

Mice may experience short term discomfort and stress at the time of the procedure such as the delivery of substances via the mouth (oral gavage). These effects are transient.

Weight loss is expected to occur in the context of some of the treatments. For example Dextran Sulphate will induce colitis and thus weight loss may occur due to diarrhoea/dehydration. In these cases weight loss can be managed by providing wet food ("mash") and usually animals respond within 24 hours. Animals will be closely monitored

and weighed daily.

Whole body irradiation and partial body irradiation can have several effects as it destroys proliferating cells including those of the blood system (the hematopoietic system) and intestine. Loss of these cells can lead to diarrhoea, dehydration and infection, possibly leading to death. We will use a split dose over several hours to minimise adverse effects and restrict damage to the haematopoietic and gastrointestinal systems. Animals will be closely monitored during and after irradiation, weighed daily during the first 7 days where transient weight loss can occur, and provided with mash and antibiotics in their drinking water. Any mouse showing distress will be reported to BSF staff or NVS and if necessary humanely killed. Mice given gene inducers or cell depleting substances can show modest weight loss (eg less than 15%) and minimal loss of condition. This has been observed intermittently and is transient and does not extend beyond the period of active dosing. Weight loss may occur if mice are fed a calorie restricted diet. Thus for all dietary interventions body condition and weight will be monitored and if weight loss exceeds 20% mice will be culled 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)?

The experimental protocols employed under this licence are classified as moderate. There is one main harmful effect, relating to Irradiation and bone marrow transplantation where all mice will be affected post irradiation, and require daily monitoring until the bone marrow transplant has established. In all other cases, such as administration of parasites and faecal transplants, the proportion of mice adversely affected is expected to be below 0.1%.

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

- Killed

Replacement

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

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

Our studies are interested in understanding how the host responds (appropriately or inappropriately) to infection, immunisation or during immunological disease. These questions require an in vivo model to precisely define the complex interactions between mixed cell populations that are activated in lymph nodes, respond and then move to the sites of response such as epithelial tissues via specific receptor ligand interactions. To date these cannot be accurately modelled in vitro. In terms of parasitic worm infections, none can be maintained in vitro or indeed infect tissues to mimic the interplay between responses at the infection site and subsequent response in the lymphoid tissue.

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

We are able to use in vitro methodologies that take place outside of an animal in a petri dish, in some cases, to make informed decisions as to how to progress the in vivo work. For example, by exposing cells from infected mice to antigens in vitro we can analyse the key cytokines/chemokine/anti- microbials they secrete. Likewise changes (up or down) in receptor expression both at the mRNA and protein level will enable us to make informed decisions, for instance, as to which molecules we should focus on to work out their function in the infection or disease setting in vivo.

Further, in our analyses of parasite derived molecules that modulate the immune system to date we have made extensive use of cell lines developed from the cells which form our barrier surfaces such as those that line the gut and the skin (epithelial cell lines) and macrophage cell lines to screen parasite secretions for immunomodulatory properties prior to screening them against primary cell lines. This has allowed us to Replace the use of animals where appropriate.

We are aware of the work by colleagues developing a model system where early stages of the parasite can be maintained in a piece of gut tissue outside of the body in a petri dish. This is their in vitro organoid model for live helminths but currently this is only useful for looking at early (larval) stages of infection and cannot be used to explore holistically the host immune response to infection. We will continue to monitor the development of this system in case it becomes suitable as a tool to meet our aims and objectives in the future.

Why were they not suitable?

In vitro methods can help us make informed decisions as to how to progress the in vivo work. However they cannot recapitulate fully the host parasite interactions that occur in vivo. Further the parasites we work with are still unable to complete their life cycles in vitro and in order to understand the host immune response to infection an animal model (the host) is essential.

Since our current project licence was granted there have been no new non-animal alternatives that have become available that we are aware of. As mentioned above the in vitro organoid model for live helminths is currently only useful for looking at early (larval) stages of infection.

Reduction

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

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

To successfully achieve the objectives of the programme of work it is important that enough animals per time point are used to achieve significant, meaningful results. We have received advice on the proposed experimental design and methods of analysis of the results from statistical experts. We have provided data, for example numbers of parasites and levels of antibodies to show how variable the data sets typically are, and these have been used to calculate the group sizes we will need to use in order to see significant differences where significant differences exist.

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

For in vivo experiments my staff use the NC3Rs Experimental Design Assistant as well as consultation with an external statistical consultant.

Egg doses for infections are carefully monitored over several years to optimise the numbers of healthy worms generated whilst minimising any adverse effects from a high worm burden. *T. muris* does not require continual maintenance in mice with infective eggs stored for years at 4 degrees. Thus to keep the parasite in the laboratory mice need only be infected when there is a need to generate more parasite eggs or parasite derived products.

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

One of our key technical goals is minimisation of variance; this is pivotal in determining the number of animals required to demonstrate biologically relevant differences (supported by statistically significant changes). Steps used to minimise variance within our models include.

Use the same strain of mice, and purchase from a single supplier with minimum variance in weight (16-18g at delivery 22-25g at experiment).

Use as far as possible the same egg batch to establish parasite infections
Prepare the substance to be delivered the same way in each experiment.
Design model endpoints to occur at times when variance is minimal
Study multiple compounds within one experiment, minimising the number of untreated control groups.

Use SOPs, to ensure comparability between operators and studies.

Calculate sample size based on available data before the experiment.

Use explicit inclusion and exclusion criteria.

Use randomisation of treatment and controls.

Where possible we blind treatments; worm burden analyses are always carried out in a blinded fashion.

To increase the efficiency of our mouse breeding colonies where possible we use of male and female mice to both avoid culling surplus stock and to better model the variation experienced in the real world.

We always try to share our mouse colonies with other researchers.
During experiments we harvest and archive multiple tissues from any one animal for future analyses, and where possible we share experiments between researchers asking different questions within the same experimental setup.

Refinement

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

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

The choice of animal model is the laboratory mouse.

Our studies focus on the use of the laboratory mouse. The three gut dwelling worms used in this project are all models of human disease. For example, Trichuris muris in the mouse is a validated model of Trichuris trichiura infection in man. Importantly the mouse species of Trichuris is remarkably similar to the human species of Trichuris at the genetic, antigenic, and physiological levels and also triggers similar immune responses in its host. Thus the mouse model of human trichuriasis enables us to develop new therapies to treat Trichuris trichiura in humans. Heligmosomoides is a rodent hookworm modelling hookworm infection in humans; and Trichinella spiralis is a worm that infects both humans and mice.
Notably, *T. muris, T. spiralis and H. polygyrus* are all natural parasites of mice and thus have little impact in terms of lasting harm to their host.

Modelling the human disease: The relevance of the mouse model to infection in man is important. In the context of immunity to infection, we, and others have shown that a Type 2 immune response is required for worm elimination in the mouse. Likewise a similar Type 2 associated protective immune responses in man. Although it is possible to study immunity to infection in man the infection status and infection history of the study population is usually undefined and longitudinal studies are difficult.

Ethically, studies are restricted to analyses of peripheral blood cells, few of which will be parasite specific effector cells as such cells are stimulated in the local draining mesenteric lymph nodes and then home to the site of infection, the gut. In addition, differences in exposure and diet all compound to make it hard to achieve immunologically meaningful results in the field.

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

Our work focuses on understanding the way a host responds to infection both in terms of immunity to infection and how infections are regulated by the immune system. In both instances we need to study the fully mature adult immune system and thus need to use adult stage mice for our experiments.

T. muris, H. polygyrus and Trichinella spiralis need to live inside the mouse host in order to complete their life cycles. T. muris for example takes 35 days to develop from the infective egg stage through to the adult stage of the parasite and therefore infections cannot be performed under terminal anaesthesia or at an immature life stage.

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

Minimising animal suffering: It is our aim to reduce any excessive suffering or pain experienced by the animals (Refinement) and to apply appropriate humane endpoints (as detailed in the protocols). There is regular close monitoring of all animals by scientific and animal care staff on a daily basis. Many of the experiments will involve only mild intervention protocols and be of a limited duration. We will always adopt the least harmful approach necessary to meet the objectives of the work.

When we combine our vaccine with certain substances, the substance can boost the effects of the vaccine making them stronger. Such substances are call adjuvants. For our vaccination studies we have only used formulations that minimise the risk of any harm. Freund's complete adjuvant is very good at boosting immune responses.

However it is made up in part of bacterial components and can cause side effects such as ulcers at the site of injection. Thus we will exclude the use of Freund's complete adjuvant and only use less reactive adjuvants typically already licensed for use in man, such as alum. The protocols employed are well established in our lab and designed not to induce suffering in animals.

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

We will follow the PREPARE guidelines (https://norecopa.no/prepare) for all our experimental work and design experiments so we can use ARRIVE guidelines (https://arriveguidelines.org/) for publications. In addition, prior to any animal studies we will prepare and submit a full experimental study plan to the animal unit to ensure all studies are carried out in line with best practices. We will conform to the principles described in the Working Party report "Refining procedures for the administration of substances" in Laboratory Animals (2001) 35, 1-41.

Use of male and female mice: when growing adult stage parasites in vivo we prefer to use male mice as they tolerate the infection better than females. In situations where we need to use female mice to minimise harm we will only use mice above 22g weight.

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

We will stay informed of 3Rs advances through:

- NC3Rs newsletters.
- Animal unit newsletters.
- Discussions with other in vivo researchers.
- Seminars put on through the animal facility.
- Any changes to best practice will be discussed with the NACWO and implemented where appropriate.

18. The role of the environment in headache and oro-facial pain 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

Environmental exposures, Headache and migraine, Sensory nervous system, Circadian biology, Inflammation

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim of this project is to understand how the environment such as light-dark cycles or pro- inflammatory diets may lead to chronic headache and oro-facial pain disorders or affect treatment response. Oro-facial pain disorders are conditions such as trigeminal neuralgia, where pain is localised to the face or mouth. By discovering the underlying molecular and cellular mechanisms, this will yield new drug discovery starting points.

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

Why is it important to undertake this work?

Headache and oro-facial pain disorders are a huge burden for society. Treatments are not good enough and difficult to develop. Although the genetic predisposition is an important factor, in many cases these conditions will only manifest after particular environmental exposures such as the type of diet we have or our sleeping patterns. Our mechanistic understanding of how the environment leads to disease manifestation is very limited. In this project we will be studying the internal environment such as hormonal and inflammatory changes and the external environment such as light conditions and diet. This is a significant bottleneck in the development of relevant translational disease models, for the discovery of druggable targets and for developing precision medicine approaches. Through undertaking this project, we hope to overcome the bottlenecks and help address the largely unmet medical need for patients with these disabling conditions.

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

The main outputs of this project will be new information on the mechanisms of headache and oro-facial pain which will be published. The specific outcomes will include: Determining how environmental factors that have been identified from patient experiences and clinical or epidemiological studies are involved in headache and oro-facial pain - from molecules to cells to nerve circuits to behaviour.

Identify the molecular mechanisms by which these environmental factors regulate the function of different cell types involved in nerve pathways involved in headache and oro-facial pain.

Investigate how different parts of the headache pain pathways may interact with other brain regions or other body systems to affect headache and oro-facial pain conditions.

Discover and validate new targets for headache and oro-facial pain treatments

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

There will be short and longer term benefits of our project. Research data generated and protocols of methods will be presented at conferences and through open access journals which will, in the short term, help to corroborate other studies in the same research area or enable the generation of new hypotheses. Better understanding of how pain varies under different environments or after particular exposures will help other researchers perform more reproducible experiments.

We will corroborate findings from this project with experiments conducted in human stem cell models. Importantly this will help establish the validity of the human stem cell models by demonstrating the importance of conserved mechanisms in regulating pain behaviours. This research will therefore support the replacement and reduction of animal research for pain in the mid-term

Longer term, the project will provide understanding of pain regulation mechanisms. This will in turn lead to targets for pain drug discovery. Academic researchers and researchers based in the Pharmaceutical industry will be able to use our data and our models through research collaborations to progress these outputs for the development of new pain treatments.

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

The project outputs will be disseminated through departmental meetings, national and

international conferences, and through open access publications. Publications will be actively encouraged irrespective of 'negative' findings in appropriate journals. We have several collaborations and knowledge arising from this project will be shared with our collaborators.

The molecular data and associated meta-data, we generate will be deposited in accessible data repositories. This will allow other researchers to make use of our data.

Tools we generate such as those that can label particular types of cells will be shared with the international research community through resources such as AddGene.
We will work with colleagues in industry in order to identify, validate and develop drug targets.

We will link to national or global initiatives such as the MRC Advanced Pain Discovery Platform to maximise research outputs.

Species and numbers of animals expected to be used

- Mice: 24000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 being used in this project because the anatomy, physiology and regulation of their pain sensory system involved in headache disorders, has many similarities to humans. There has already been substantial investigation of the mouse with respect to circadian biology, metabolism, inflammation and pain biology and this means that many tools are available to achieve our project outcomes. In particular there is an extensive set of genetic modifications that allow identification and modification of activity of specific cells or nerves that might contribute to pain regulation. It is important to determine how these mechanisms ultimately determine sensory and pain behaviours and the mouse provides a way to assess this.

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

Genetically altered animals will be bred, which will not cause any harms. Some of our mice will have genes that are not active until we choose to activate them with an injection of a substance such as Tamoxifen. This removes the need to breed mice with harmful phenotypes

Typically, animals will experience mild, transient pain and no lasting harm from administration of substances using standard routes such as intraperitoneal or oral. They will typically have behaviour tests to assess their sensory thresholds and pain sensitivity. The circadian rhythms of the mice may be altered by maintaining the mice under altered lighting conditions or by changing feeding schedules.

Metabolism can be altered through changes in diet. Inflammation can be induced transiently and reversibly through substance administration. Blood samples may be

collected before and after substance administration or altering their environment. Typically these experiments may last 4-8 weeks.

Some animals may have brain or spinal cord injections to turn on or turn off a particular gene. This will be carried out under general anesthesia. The mice may have some mild to moderate pain on recovery from surgery, which will be managed with analgesics. After recovery these mice may have administration of substances, blood sampling, alteration of their circadian rhythms and then testing of their sensory behaviours. These experiments, including the initial surgery, may last 8-12 weeks.

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

The genetic alterations are mainly to identify particular cell types or to alter their function in ex vivo experiments so are not expected to cause any harm.
Substance administration and some genetic alterations may alter pain sensitivity. If sensitivity is expected to increase then experiments will be adjusted to prevent unexpected pain or distress.

Administration of substances such as lipopolysaccharide (LPS) may induce a short period of sickness, such as experienced by humans after vaccinations, and this is not expected to last more than 2-3 days. Animals will be closely monitored during this time for weight loss or other signs of distress.

Alteration of the circadian rhythms through change in feeding schedules, sleep deprivation or altered light exposures is not expected to have any lasting impacts. Animals will be monitored for signs of distress or weight loss. Similarly changes in diet is not expected to cause lasting harm and animals will be monitored closely for weight loss or signs of malnutrition.

Animals undergoing surgery may experience pain and discomfort during the recovery period. This will be managed through use of analgesics before and after surgery.

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

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

70% subthreshold
15% mild
15% moderate

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

- Killed

Replacement

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

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

We are looking to understand the interaction between the environment and the pain sensory system - in particular the pain system for the head and face. This requires an intact whole organism to study since there are multiple ways that these two systems can interact. Using animals we can test interventions to confirm causal relationships. We also need to understand how the mechanisms change behaviour, which is possible because the mouse has a fully developed sensory system.

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

We extensively use human stem cell models to understand pain mechanisms. Stem cells are able to be turned into any cell type in the body which allows us to study how particular cells might be involved in conditions such as headache and pain. A particular type of stem cell are induced pluripotent stem cells that are made by taking blood cells from a patient and turning this back to a stem cell. For our research, we generate pain sensory neurons as well as other cell types that may be involved in pain regulation. This will be ongoing and will complement the animal studies in this project enabling reduction and eventually replacement.

Why were they not suitable?

The models are a powerful means to investigate molecular and cellular mechanisms but they cannot presently reconstitute the organism level interactions that likely underlie regulation of pain.

Furthermore it is necessary to understand how these mechanisms change pain related behaviours, which therefore require 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 numbers based on our previous experience with similar experiments which have been published as well as published work by other groups. Our experiments are designed to ensure sufficient animals to detect a biological difference, taking into account the variance of the experiment and the size of the effect. These can vary depending on the experiment and in particular whether the mice are undergoing behavioural tests in which case typical sample sizes are 12-16 animals per experimental condition, where we ensure an even mixture of male and female mice. A typical experiment may then comprise 2-4 experimental conditions.
We have also estimated based on past experience, including previous home office returns, the number of animals required for breeding of genetically altered animals to produce offspring with the desired genotypes. This is also based on expected inheritance patterns as well as mating strategies and colony size requirements to undertake the proposed experiments.

The overall numbers for the project have then been estimated based on the time scale of a 5 year programme and the number of researchers likely to be working on this project over this time.

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

- We will follow ARRIVE guidelines and have taken the following steps to reduce the number of animals
- Initial in vitro studies to develop hypotheses and plan in vivo validation
- Preliminary in vivo studies to identify optimal sample sizes given experimental noise and effect size
- Factorial design
- Multiple end points in each animal where possible
- Review outcomes for significance to ensure optimal experimental design

Additionally our experiments will balanced for sex to ensure that experimental outcomes are relevant to males and females. Experiments are performed blind to genotype or where possible experimental condition. Animals will be randomly allocated to treatment group or experimental condition using the NC3R's Experimental Design Assistant. Randomisation and blinding will reduce bias and avoid the need to repeat experiments.

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

Colonies will be maintained efficiently with a planned breeding strategy to produce the animals required for experiments in a timely way whilst minimising the numbers required. Through the use of a mouse colony management system we will ensure only animals needed for experiments are produced. Breeding colonies will be managed in line with the best practice guidelines.

The genetically altered mice used in this project are on bred onto the same background strain which will help reduce variability in experiments. We will also use littermates as controls, which avoids cage related biases. Tissues from mice in experiments will be stored if not immediately used and can then be shared with colleagues or used for later experiments.

Experimental conditions will be optimised for example ensuing animals are familiarised with the operator and the testing environment to reduce variation.
Pilot studies will be undertaken for newly designed experiments to understand variance and optimise the experimental design.

Refinement

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

Which animal models and methods will you use during this project? Explain why

these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use genetically altered mice where genes involved in circadian regulation may be deleted from specific cell types in the somatosensory system. By restricting the modification of a gene to a specific cell type, this minimises adverse effects and helps us understand the specific role of the gene in pain mechanisms. In most cases these mice are healthy and show normal behaviours. In some cases we will use global deletion of a clock gene but this is required to contrast with the specific deletion to interpret our findings. However there is considerable redundancy in clock mechanism which means that suffering and harm is minimised.

We may also use genetically altered mice to modulate activity of a gene involved in a pain mechanism. This may result in mice either more or less sensitive to pain. Where this is identified, we will refine our experimental parameters to change the strength of the stimulus when testing for sensory thresholds to ensure they are never exposed to intolerable stimuli. We do not anticipate these mice will show any lasting harm or distress.

We may administer substances through various routes to then test how these affect pain sensitivity. We will use the most refined route of administration, for example implanting an osmotic mini-pump if repeated and prolonged administration is required. Any change in pain sensitivity arising from substance administration is transient and reversible. We are interested in the process where transient pain becomes persistent pain, known as chronification and we have found that alternate day administration of a substance such as nitroglycerin or CGRP is sufficient to reduce pain thresholds, without the requirement for daily injections.

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

Our goal is to translate our research on environmental factors into new treatments. This means the use of non-mammalian animals are limited because their pain systems are not sufficiently similar to produce findings that will be relevant for humans. The genes involved in regulating pain biology in less sentient organisms may have different functions or may not even be present. For a similar reason we will use adult mice since key genes may not yet be expressed and systems that mediate environmental effects such as the circadian system or metabolic pathways also takes some time to properly establish. We cannot use terminally anaesthetised animals as we need to demonstrate causal relationships between hypothesized mechanism and somatosensory behaviours.

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

In this project we will undertake a continuous process of refinement of procedures. This will include minimally invasive methodologies and least stressful behavioural and physiological assessments. Suffering will also be minimized through appropriate use of analgesics (dosing based upon our prior experience of achieving effective analgesia) when undertaking surgeries which will employ aseptic surgical technique. Analgesics will be provided prior and after surgery.
The animal models used to investigate pain sensitivity will be refined to minimize stress and suffering whilst ensuring robust outcome measures with changes in sensory thresholds. For these behavioural tests, the animals will be familiarised with the experimental apparatus and also with the operator.

Orofacial sensory assessments will be carried out with apparatus using no restraint to the animal. We will assess responses to threshold natural stimuli (for instance reflex withdrawal to a heat stimulus) rather than subjecting animals to persistent, high intensity unavoidable stimuli. The frequency of behavioural testing will be reduced and undertaken at key time points and combined with obtaining tissue to enable multiple experimental outcomes.

For circadian experiments, animals are routinely group housed in ventilated and sound-attenuated light tight chambers (LTCs) in which the light environment can be carefully manipulated. This also enables us to collect various physiological and behavioural data without disturbing the animals. Where the light environment is experimentally manipulated within LTCs, light intensity is closely monitored below the levels which would result in retinal damage. We will investigate remote video monitoring as a more refined method to assess activity patterns.

Where intervention such as surgery or a new substance administration or gene manipulation might increase the risk of adverse effects, monitoring of animals will be more frequent to assess for any unexpected pain, suffering, distress or lasting harm.

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

All experiments will be designed, performed and reported in accordance with the ARRIVE and LASA guidelines (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?

Attendance at animal welfare meetings within the establishment.
The National Centre for the Replacement, Reduction and Refinement of Animals in Research (NC3Rs) website provides a comprehensive resource for protocols and the latest developments in the 3Rs in the UK.

All licensees will be encouraged to access the NC3Rs resources, and experiments will be designed and enacted using the NC3Rs Experimental Design Assistant.
Our institute also has an NC3R’s regional manager available for consultation, along with regular internal 3R’s meetings.

19. Understanding the impacts of inhaled particulate matter on neurological health

Project duration

5 years 0 months

Project purpose

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

Air pollution, Brain, Inhalation, Particulate Matter (PM), Neurological

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The proposed project will employ rodent models for a comprehensive investigation into the impact of inhaled particulate matter (PM) on neurological health, including the characterization of pollutants from different sources and assessment of their effects on the blood-brain barrier (BBB), examination of particle deposition in the brain, identification of biomarkers for neurological disorders, evaluation of how material properties influence neurological disorders, and exploration of the underlying mechanisms of neurological effects and behavioural changes, all with the goal of enhancing our understanding of the relationship between particulate pollutants and brain health.

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

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

Why is it important to undertake this work?

Worldwide, around 50 million people live with dementia and this number is expected to triple over the next 30 years, representing a significant population health challenge. It is estimated that the economic impact of dementia is greater than the combination of cancer and heart disease. In the UK, the total cost of dementia, including costs to the NHS, paid social care and unpaid care, has risen to £34.7bn and will rise further to £94.1bn by 2040. In the 2020 update report of the Lancet Commission on Dementia Prevention, Intervention, and Care, air pollution was one of the three newly added modifiable life-course risk factors for dementia. The aetiology of dementia and other neurodegenerative disorders are complex and not fully understood, but accumulating epidemiological evidence has shown that exposure to air pollution, especially ambient particulate matter (PM), is an important environmental risk factor for neurodegenerative and other neurological disorders (PMID: 25734425, 33340865). Children have been considered to be more sensitive than adults to PM exposures, due to the greater absorption in the tissues of the head, and a longer lifetime of exposure. Children from Mexico City had substantial cognitive declines as compared to population norms, and as compared to other children of similar age, sex and family and neighbourhood backgrounds who lived in less polluted areas (PMID: 32006765).

By examining brain tissues from young adults and dogs after unexpected sudden “accidental” deaths, evidence has shown that there is a connection between early signs of neurodegeneration and air pollution (PMID: 18349428, 28598844).

Despite growing epidemiological and toxicological evidence, the biological mechanisms by which air pollution could impact neurological health are currently uncertain. Given the significant impact of brain diseases on individuals, the population at large and the economic burden of neurodegenerative diseases, this is considered an area worthy of investigation.

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

The primary scientific outputs from the project will be:

- The identification of the potential effects of inhaled particulate pollutants (such as those derived from combustion engines, woodfires, or engineered nanomaterials contributing to particulate pollutants) on neurological health and susceptibility to diseases, with an initial emphasis on examining their biological impacts on the blood-brain barrier (BBB).
- The discovery of biomarkers associated with neuroinflammation, oxidative stress and neurotoxicity, and the potential translocation of inhaled particles, and particle associated toxicants across the “lung-to-brain axis”.
- Enhanced insight into how the physico-chemical properties of particulate matter (PM) and pollutant materials influence neurological health and susceptibility to neurological diseases.
- A comprehensive understanding of how exposures to particulate matter (PM) and the underlying cellular responses impact neurological disorders, cognitive decline and animal behaviour.

The scientific findings will be disseminated widely. The data and findings will be made available as appropriate through a variety of different outputs, which will include peer

reviewed publications, expert reports and presentations at scientific conferences as well as public health advice. Such outputs will be accessible by other researchers (within the UK and around the world), policy makers, regulators, health providers, patients, and the general public. The collaboration with Air Quality and
Public Health Group will enable relevant findings to be fed into working groups on air pollution, COMEAP and policy discussions.

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

There is growing evidence on the association between air pollution and mental health, cognitive decline and neurodegenerative diseases, which becomes one of the most significant recent developments in our knowledge of the way that air pollution may affect public health. Our current lack of understanding about the mechanisms by which air pollution may impact neurological health limits our ability to understand the overall extent of harm resulting from air pollution and design effective policy and interventions to address this. Results from the proposed research to understand how particles impact on the brain will provide important background information to support our advice-giving role. Together with information from cohort studies, exposure studies performed on human beings and similar studies in other laboratories, these studies will help to improve the evidence on which to base limits and guidelines for human exposure as well as advice for policy makers nationally and internationally. We also envisage our data outputs will contribute to the development of new scientific approaches aimed at understanding the detrimental health effects of particulate pollutants through the development of adverse outcome pathways (AOPs), markers of exposure/toxicity, establishment of non-animal exposure models linking the respiratory and neurological systems, and, in general, new approach methodologies (NAMs) for risk assessment and regulatory purposes. The data will also potentially contribute to the development of mitigation strategies to reduce harmful effects.

Direct short-term benefit comes from the improved understanding and increased evidence of the impact inhaled particles on the neurological health, and scientific disseminations including publications, conferences etc. The indirect benefit, including the impact on influencing the related policy and contribution to advice, guidelines, exposure limits and strategies, would come later.

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

This proposed project will combine expertise in both lung and brain toxicology, involving members who have significant expertise in in vivo and in vitro inhalation toxicity studies to understand the health impacts of different airborne contaminants, and members who have significant experience in investigating neurological effects of different toxins. The proposed project represents another opportunity for further collaboration and combination of expertise in this research project, e.g., supplying tissues for downstream further analysis such as histopathological analysis, RNA- sequencing, epigenetic analysis etc. All these would make important advances in the field of inhalation toxicology of air pollution so to be considered to improve the outputs of the work.

Wide dissemination of the results, will be achieved through publication in peer reviewed journals, facilitated by the strong working relationships between different department and relevant policy makers (e.g. Defra) and the wider Air Pollution research community for wider scientific dissemination, and in collaboration with networks' wide ranging activities and identify (e.g. UK Clean Air community) to enable the findings of the proposed project to be fed into working groups on air pollution, COMEAP and policy discussions etc.

Species and numbers of animals expected to be used

- Mice: 400
- 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.

Toxicological approaches using animal models would be the most appropriate scientific approach since animals will provide a better model of the situation that may occur in exposed humans and animal exposure would be the most accurate models of mimicking human exposure and/or physiology. Rodent models (rats and mice) are chosen for these studies as they are the lowest sentient animal, with a complete immune system that closely mimics the human circulatory/respiratory system.

Performing these exposure studies in rodents ensures the proposed project to address questions on systemic toxicological effects linked between two biological systems - respiratory and neurological. Therefore, classic toxicological approaches focused on the exposure of animal models will be applied. Rats in addition to mice are chosen for this project as they are considered more sentient, which is particularly useful to explore the biological mechanism interlinking the neuroinflammation and/or oxidative stress that are induced by inhaled PMs and development of neurodegeneration and cognitive declines.

Rodent models are selected also for historical reasons since we have extensive experience of a wide range of rodent models of various strains and disease models, especially on inhalation assessment through different pulmonary exposure pathways. We also have substantial experience with the generation, characterization and the administration of chemicals and/or particles to the lung and the subsequent monitoring of adverse effects. As such, we have developed direct relevant experience for the proposed work in this new project application and built collaboration with colleagues with extensive experiences in neurobiology to provide additional support and expertise. All these would allow us to assess the neurological effects after exposure to particles.

Rodents from juvenile through to adult will be considered for exposure studies using animal models as evidence has shown that there is a connection between air pollution and early signs of cognitive decline and development of neurodegeneration.
The decision to use both rats and mice within this proposal is based on the specific research objectives considering the characteristics of each species and ethical concerns. Rats and mice, being closely related rodents, share many similarities in terms of brain structure and physiology. However, there are also some differences, primarily related to size and certain aspects of their brain morphology. Rats have larger brains compared to mice due to their larger body size. This increased brain size can provide more space for neural structures and potentially more complex cognitive capabilities. Possibly due to this reason, rats are often preferred in studies related to complex learning and memory tasks due to their larger brain size and longer lifespan, which may allow for more extended experiments.

Mice, on the other hand, are commonly used in genetic studies due to their well-

characterized genomes and ease of genetic manipulation. Moreover, rats typically have larger olfactory bulbs compared to mice, in proportion to their overall brain size. The larger olfactory bulbs in rats may be related to their potentially more complex olfactory capabilities. While both species have highly developed olfactory systems, rats are often considered to have a more acute sense of smell compared to mice. Rats rely heavily on their olfactory abilities for navigation, communication, and foraging.

Particles could be potentially translocated into the brain through the olfactory bulb pathway as well as translocation from blood circulation. Therefore, studies as proposed using rats would cover more information for the pathway through olfactory bulb. While mice are generally considered to have lower sentience, there are specific scientific reasons for need both rats and mice. When it comes to modelling certain neurological disorders, the efficacy of studying specific genetic mutations is enhanced in mice given there are more options for gene modified models in mice, especially those closed to Dementia disease models. Additionally, for studies that involve correlating imaging (examining particle translocation to the brain and assessing histopathological effects in the brain) with transcriptomic analysis and behavioural assessments, mice provide a more effective model.

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

Typically, animals will experience mild, transient pain and no lasting harm from administration of substances by inhalation/instillation/injection using standard routes. Some of the animals may experience some discomfort at the beginning of exposure (nose-only exposure) or after instillation/injection, and some mild to moderate pain which will be treated with analgesics if required. Some of the animals will be pre-treated with certain chemicals (e.g., toxin administration to introduce the condition with increased BBB permeability; dye administration to determine BBB integrity) to facilitate the detection of particles deposited or transported to the brain and to assess potential harm to specific brain regions. Animals may experience some mild to rare moderate discomfort during behavioural assessment. Animals may experience mild and transient discomfort from blood sampling or urine collection. 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.

Behavioural assessments may be carried out prior to particle exposure and repeated after exposure to assess whether exposure impacts cognitive or motor functions. Tests are optional but three main aspects will be considered including social aspect performance, leaning/trained behaviour/memory recall, and exploratory behaviours etc. Options of social Interaction tests include tube dominance test, three-chamber sociability and social novelty preference test. Optional tests for learned/trained behaviour include Barnes maze and Y maze. Optional tests for exploratory behaviours include novel object recognition, open field and marble burying test. Other behavioural testing, such as assessments linked to award & addiction may also be considered to examine the effects of exposure in rodents.

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

Adverse effects may vary depending on the procedures, the strain of rodents used, and the time-point at which the animal is being investigated.

Exposure related effects: Animals may exhibit stress response to restraint, but this will be minimized by habituation to the equipment prior to exposure or dosing.
Pilot studies will be performed to identify safe and appropriate dose rates. A small portion

of animals may have up to moderate adverse effects, including potentially developing systemic toxicological effects post exposure, e.g., weight loss over 10% within 24 hours etc. Animals will be monitored and killed if humane endpoints are reached.

All the behavioural tests are selected for the specific scientific objectives and logistical considerations. Behavioural assessment related effects: In general, there are mild effects on animals during behaviour assessments. Animals may respond with stress during testing, for example during the tube dominance test and the three-chamber sociability and social novelty preference test, although it is very rare.

Urine collection and blood sampling related effects: In general, there are mild effects on animals during these procedures. If animals will be housed in pairs or threes in cages for this purpose, urine will be pooled to generate appropriate sample groups for analysis. Males can't be grouped together from different littermate/cage and single housing will be avoided in cages where possible.

Age and strain related effects: We plan to use some rodent strains which have been used in other behavioural studies in the literature, such as Long Evans rats, C57BL/6J mice etc. For behavioural assessment on rodents for mid to long-term (over three months, up to fifteen months) post exposure duration, selection of strains will be based on their application in similar studies in the literature and avoidance of any specific potential age/strain related effects. Strains of rodents will also be considered based on their feasibility for the specific behavioural tests.
Expected severity categories and the proportion of animals in each category, per species.

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

Mice: 25% mild, 75% moderate;
Rats: 25% mild, 75% moderate.

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

- Killed

Replacement

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

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

We need to use animal models to understand how inhaled particulate matter (PM) impacts the neurological health. There are mainly two hypotheses as to how air pollution might manifest its acute and chronic impacts on the brain. The first is direct interaction of inhaled particles, or desorbed chemical constituents, with non-neuronal glial cells and neurons in the brain, based either on their uptake via translocation along olfactory neurons to the olfactory bulb, or their entry across the blood brain barrier (BBB) from the circulation; the other hypothesis is indirect impact that air pollution induced systemic inflammation and its transmission to the brain across the glial-neurovascular unit. It is essential that models allowing systemic distribution are to be used. In this way, they will provide a better model of the situation that may occur in exposed humans. The biological mechanisms are

complex and not fully understood. Thus, no in vitro system is currently able to replicate this.

To address questions on systemic toxicological effects linked between two biological systems, herein respiratory and neurological, classic toxicological approaches have been focused on the exposure of rodent models. Rodents have been chosen for these studies because they provide a very good model system to study the systemic effects including the brain. There is a wealth of biological data concerning rats and mice, and we have extensive experience of using these species in similar studies for over ten years. Furthermore, using rodents would maximize the potential for use of genetic markers and the potential for interpretation of results using genetic databases.

In order to evaluate the age-related and other effects of particle exposure on brain physiology and behaviour, it is essential to use aged alert, naturally-behaving animals that show the full repertoire of integrative functions.

Animal models also have the advantage that numerous biological endpoints can be obtained from a single animal. It is essential to use animals to study the consequences of in vivo exposure in the gene and/or protein expression studies following particle exposure profiles. These in vivo gene and/or protein expression studies will be supplemented by and correlated with complementary in vitro studies where appropriate.

Overall, the data obtained with animal models are far more valid for health risk assessment than that provided by any alternative models, and they allow clearer extrapolation to the potential risks in humans. We will however keep informed about other models available for particular genes of interest or gene pathways that maybe identified throughout the course of the research. We will also be developing non-animal models to support a transition away from traditional animal testing and to facilitate hazard identification for airborne contaminants and health risk assessment of inhalable materials.

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

In other complementary work in our group, some in vitro experiments have started. Human relevant in vitro exposure models could capture organotypic functions as well as mimicking realistic exposure, therefore we have been continuously using and optimizing in vitro models (animal and human cell- based models) of exposure for the assessment of biological responses to inhaled particles. As experiments in animals cannot capture all the biological consequences of public exposure to air pollution at cellular levels, a non-animal alternative option is to develop a BBB exposure model as the gateway for neurotoxicity assessment.

It is hoped that human-relevant BBB models will be established and optimized, which could be used to investigate the effects of particles from blood circulation on the brain. Ex vivo BBB models will also be considered to investigate the effects of particle exposures to study molecular and cellular mechanisms which may ultimately lead to the discovery of potential therapeutic targets and may reduce the number of animals used. These in vitro and ex vivo studies are intended to complement the animal studies since behavioural endpoints can be correlated with neurotoxicological endpoints, e.g., neuroinflammation, oxidative stress, morphological changes in neurons and microglia etc.

Previously we have established and optimized an aerosol exposure at air-liquid-interface (AE-ALI) system that mimics a realistic human exposure scenario and captures biological responses including inflammation and oxidative stress etc. Detailed characterization of this

exposure system has been performed considering several operational and biological parameters. Validation on the relevance, reproducibility and predictivity of this exposure system has been recently completed to facilitate more realistic hazard identification of airborne contaminants in human relevant culture models. However, this AE-ALI system is complex to use, and improvement of the system is necessary to fully reflect the complexity of the respiratory system. Depending on the feasibility, linking the BBB exposure to the AE- ALI exposure system might be a promising approach for non-animal alternatives, which will be considered.

Why were they not suitable?

It is not feasible to use in vitro models, non-sentient alternatives or to use lower organisms, such as invertebrates, plants or micro-organisms, or to use vertebrates at an earlier stage of development. We need to use alert, naturally-behaving rodents that show a complete range of bodily functions, such as thinking, learning, sleeping, moving, eating and drinking. However, throughout the project duration, we will regularly review whether alternatives might be appropriate and incorporate them where appropriate.

It is not possible to investigate the effects of early life exposure using in vitro methods or in laboratory studies with volunteers for differing ethical reasons.

BBB models could only cover a limited part of the pathways by which inhaled PM could impact on the brain, including disruption of the BBB integrity, the potential translocation of particles from systemic circulation to the brain etc. Some parts of the pathways by which inhaled particles could impact on the brain are missing, such as olfactory bulb pathway, the interaction with the respiratory tract, potential translocation from the pulmonary epithelium to the blood, bioavailability of the particles within the blood circulation, involvement of other brain cells (e.g., microglia, glia cells, neurons) etc.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 follow the NC3Rs' experimental design guidance and employ experimental design assistant (EDA) to plan our experimental design, practical steps and statistical analysis utilizing the advice and support for randomization and blinding, sample size calculations and appropriate statistical analysis methods.

For both inhalation (nose-only inhalation and whole-body exposure) and instillation (intranasal instillation and intratracheal instillation) procedures in rodents, previous work within our group and other studies in the literature has allowed us to determine the minimum number of animals required for each treatment group for both inhalation and instillation procedures in rodents. Prior to designing the project, a literature search was carried out. Up to date, 39 publications on neurological health with a focus on brain deposition or neurotoxicity effects of inhalable substances related to air pollutants using animal-models have been identified. These studies were assessed in detail with conditions

and other specifics of the experimental set-up collated as reported. The purpose of this literature review is to pinpoint areas where knowledge is lacking so that we can strategize our experiments and minimize the utilization of animals in our research endeavors. More detailed statistical analysis, including power/sample size calculations will be used to determine minimum numbers for each experimental study.

When comparing adverse effects of different particulate material types, a randomized block design approach will be used to increase experimental precision and reduce the number of control animals needed for comparison. Treatment groups will be always blinded, and sample labelling performed by someone not directly involved in the experimental work. Appropriate parametric or non-parametric statistical tests will be used depending on data distribution. Likely statistical testing involves an assessment of data distribution and parametric comparisons of sample groups using two-sample t tests (for two sample groups) or ANOVA for more than 2 sample groups.

We will only use sufficient animals to meet our planned experimental needs. For qualitatively imaging analysis, it is estimated that less than 100 animals (50 mice and 50 rats) in total will be used. For quantitative analysis including the behavioural assessment, it is estimated between 6-9 animals will be used per treatment group and typically to around 40-60 rodents of one sex being used in one exposure study. This is based on previous experience as well as a study of six groups for one type of particles, including two control groups, two exposure doses and two time points post exposure. Therefore, it is estimated maximum 120 animals (60 mice and 60 rats) per year will be used for in vivo studies if one experiment is carried out per year.

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

We will follow the NC3Rs' experimental design guidance and employ the NC3R's EDA to plan our experimental design, practical steps and statistical analysis utilizing the advice and support for randomization and blinding, sample size calculations and appropriate statistical analysis methods.

Experiments will be designed so that they can be published in accordance with the NC3Rs ARRIVE Guidelines. This includes randomization of exposure groups and blinding of samples prior to analysis to prevent bias.

Experiments will be performed following a system of written protocols and/or optimized standard operating procedures and will include positive controls where appropriate. These not only ensure consistency and reproducibility in the results and help to reduce unnecessary variability. In turn these should help to reduce the numbers of animals needed. The use of healthy animals, obtained from a reputable registered supplier, should also help to avoid loss of experimental animals through disease.

We will also commit to the PREPARE ((Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines. This includes: I) completing the 2-page checklist covering three aspects (1. Formulation of the study; 2. Dialogue between scientists and the animal facility; 3. Quality control of the components in the study) so to improve the quality of the preparation for each animal study; II) keep updated with the comprehensive website (https://norecopa.no/prepare), with detailed information and links to global resources.

What measures, apart from good experimental design, will you use to optimise the

number of animals you plan to use in your project?

The numbers of animals used will be reduced as far as possible following the NC3Rs' experimental design guidance and employ experimental design assistant (EDA). The use of replicates and appropriate control groups are necessary for endpoints that have not been previously studied, to avoid the possibility of spurious results and ensure appropriate statistical power. This is especially relevant when studying the effects of physical agents that may only produce subtle effects.

Pilot studies for some experiments may use smaller group sizes and will be used for power calculations in further studies.

Computer modelling such as Multiple Path Particle Dosimetry (MPPD) model will be applied for estimation on the particle deposition for guiding the expected exposure dosimetry and interspecies dose extrapolation.

Advanced technologies will be combined so to correlate the results. In this way, one animal would be used as its own control, and can provide data at each treatment condition, rather than a different animal being required at baseline, and for each treatment condition. This would dramatically reduce the number of animals required. For example, to identify the specific regions of the brain translocation of inhaled particles, adjacent brain slices will be processed for different staining and imaging, including ICP-MS laser ablation for observing metal-based particles, staining for identifying changes in BBB permeability, haematoxylin and eosin (H&E) staining for identifying any brain injury etc.

Multidisciplinary research is needed to address the complex health hazards posed by air pollution; therefore, multiple endpoints will be measured from same animal, including phenotypic endpoints as well as gene and/or protein expression endpoints. Animals in control groups will be reused for establishing and improving ex vivo BBB models and in vitro models based on primary cells such as BBB endothelial cells, pericytes, astrocytes and other neurological cells including microglia, neurons etc. At the end of the experiment, we will harvest as many tissues as possible at post-mortem. If we don’t need to analyse the tissues immediately, we will freeze them and make them available to other researchers working on similar questions.

Refinement

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

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

The rodents will be housed in enriched conditions with bedding material. We have optimized and refined the experimental protocols intended for use in this project, which will ensure that the number of animals used and the potential for any animal suffering is kept to an absolute minimum. However, we will continually review the scientific literature to assess whether alternatives are available, and we will continuously review and amend our

existing protocols accordingly where potential refinements may be identified.

For some investigations, some rodents may have to spend time in a separate cage for the collection of urine to enable longitudinal assessment to identify potential biomarkers of exposure/toxicity. Collection of blood sampling may also be considered for metabolomic or proteomic studies to identify any biomarkers such as corticosterone after exposure or circulating inflammatory markers e.g., Tumour Necrosis Factor-α.

A pilot study to identify the suitable exogenous tracer(s) for BBB permeability will be performed. Conditions of control and positive control (BBB permeability increased with toxin administration) will be tested, so different sizes of tracers and the intravascular infusion of these exogenous tracers could help to demonstrate the functional and structural change of BBB. Another option is to do immunostaining on the microvasculature (e.g., tight junction protein of BBB). Procedures will be optimized reusing some of the brain tissue sections from the pilot study.

Certain behavioural tests are selected based on the association between the cognitive deficits and neurological disorders, and depends on some related animal studies in the literature. Prior to designing the project, a literature search was carried out. Up to date, there are 39 publications on neurological health with a focus on brain deposition or neurotoxicity effects of inhaled particles using animal- models. These studies have been assessed in detail with conditions and other specifics of the experimental set-up collated as reported. Cognitive domains tested were largely within the three categories including memory & learning, anxiety & depression and locomotion assessment. But there is a distinct lack in research of behavioural endpoints regarding social behaviour and reward & addiction. Therefore, we will include behavioural tests from social aspects as one of the focuses.

Selection between rats and mice will be based on the practicality of the tests and techniques applied. For behaviour assessment rats would be preferred as rats are relatively easier to handle and motivate compared to mice. For example, tests may be less appropriate for assessing spatial learning and memory (i.e., learning to locate an object in relation to its surrounding) in mice than rats. For investigating/tracking the locations of particles in the brain or other associated tissues using advanced microscopy (e.g., transmission electron microscopy (TEM), scanning electron microscopy (SEM)), the choice between rats and mice will be decided considering brain size, sample preparation and feasibility for the techniques utilized.

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

Other in vivo models (e.g., D. rerio (zebrafish), C. elegans, D. melanogaster) do not sufficiently model the public health relevant issues we want to address (such as the complex interactions between the respiratory and neurological systems) compared to mammalian species. It would also be difficult to expose these models to chemicals in a way that models human exposure. Thus, while we are not currently aware of any immediate alternatives that would enable us to achieve our objectives without using animals, we will continually re-evaluate the use of potential less sentient alternative models as the proposed work, and field in general, progress.

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

For inhalation studies applying nose-only inhalation exposure route, animals will be

habituated to inhalation exposure tubes prior exposure to minimize the adverse effects. Inhalation exposure studies will only be conducted when specific scientific objectives and logistical considerations permit, given the significant time and labour requirements involved. To enhance the realism of the studies, well- characterized environmentally relevant particulate matter (PM) samples may be utilized. The aim of the aerosol production will be to achieve shorter and less frequent exposure periods. This can be achieved by increasing the concentration of the PM solution for aerosol generation using a nebulizer, improving the dispersibility of certain PM samples through sonication, or by adding certain chemicals (e.g., BSA), etc.

Drawing from past experiences in inhalation studies, we will implement careful habituation practices for nose-only inhalation exposure. Prior to the exposure phase, animals will be familiarized with the restraint process. Same or similar exposure tubes or tube-shape objects will be introduced into their cages. They will be trained to enter the exposure tubes when prompted and spend some time inside them. Based on our prior experience, rodents typically adapt well to this tube environment. To encourage this, food could be positioned near the open end of the tubes to entice the animals to place their heads inside during tubing. Additionally, animals from the same cage will be encouraged to remain in close proximity throughout the tubing and exposure process. It is expected that, as we have seen in experiments conducted to date, the animals will quickly acclimatize to restraint. Handling, loading and unloading by competently trained staff will help reduce restraint stress.

For other more invasive exposure routes or chemical administration, such as intranasal instillation, intratracheal instillation, intravenous injection, intraperitoneal injection, anaesthesia and analgesia may be utilized to help minimize the pain and distress of the experimental procedures. Proper physiological monitoring will be performed throughout the time of anaesthesia along with continued monitoring of the rodent during recovery and the study period to assess if additional analgesic intervention is warranted.

Many of the behavioural studies are undertaken using video capture and animals can be monitored remotely using this equipment. Behavioural tests will be performed and timed in a manner that is the least distressing to the animals. Animals will be limited to no more than three behavioural tests per week and also by minimizing the number of tests carried out per animal. In home cage monitoring studies, it was found that environmental enrichment within home cages and altering the bedding material to a more absorbent material helps to reduce disturbance (and thus stress) to the animals.

For urine collection, non-invasive method such as using hydrophobic sand will be utilized if more than five timepoints of urine collection. Rodents will be (single- or group-) housed in solid bottom cages with an adequate quantity of LabSand®, ~0.5 cm to cover the bottom of the cage, urine drops will be collected with a pipette (single or multiple collections) and transferred in a polypropylene tube with a cap.

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

THE NC3R's, NICE, ARRIVE, FELASA and Home office guidelines such as the guidelines laid out in the project and personal licence will be followed in order to carry out best practice.

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

All staff are committed to the 3R principles, and this will be a priority focus throughout the project activities. As the project progresses and experimental data analysed, further refinement opportunities if identified will be incorporated where appropriate in subsequent work.

During this project, we will follow the FELASA, NC3R, LASA websites and newsletters, and regularly review the literature for novel methods and techniques to improve our project in accordance with the 3Rs. We also aim to participate in regional NC3R's seminars and updates when possible and if relevant to LASA/NC3R's meetings or meetings recommended by LASA/NC3R and AWERB. We will also follow announcements from companies and laboratories who are developing lung-brain linked non-animal models with the potential aim to replace and reduce the number of animals where possible.

20. Understanding brain development in health and disease

Project duration

5 years 0 months

Project purpose

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

Key words

Neuroscience, Development, Physiology, Circuits, Neurons

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim of the outlined work is to advance our understanding of the maturation of a brain region called the prefrontal cortex. This brain region is involved in learning, memory and controlling our emotions.

Understanding the development of the prefrontal cortex is important because it will explain how our cognitive abilities improve as we grow up and give insight into diseases such as autism and schizophrenia.

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

could be short-term benefits within 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 prefrontal cortex (PFC) is an important brain region that is critical for higher-order functions, such as cognition and working memory, and has been implicated in many mental health disorders, including autism, schizophrenia, depression, addiction, and various affective disorders. As the brain develops, changes in the connections formed between neurons within the PFC contribute to improvements in our mental capabilities as we pass from childhood, through adolescence, into adulthood. The period of adolescence is thought to be particularly important, with many cellular and behavioural changes occurring at this time. If prefrontal development goes wrong, it may cause several neurodevelopmental disorders, including autism and schizophrenia. Whilst the behavioural changes that occur in patients that have disorders associated with PFC dysfunction are well characterised, we do not yet have an understanding of the cellular mechanisms causing these behavioural changes, i.e. how the neurons in the brain change their connectivity and function to cause symptoms of these disorders. This project aims to address this gap in our understanding. Given autism and schizophrenia both impact around 1% of the global population, these advances have clear significance for human health and wellbeing.

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

The work will generate data which will expand our understanding of how brain circuits are formed.

It will generate data to explain how risk factors for neurodevelopmental disorders, such as autism and schizophrenia, alter brain development.

This data will form the basis of scientific publications in peer reviewed journals and will be shared at international conferences, which will be beneficial for members of the scientific community working to understand the brain.

The data will be used to create anatomical datasets that will be shared with other researchers, free of charge.

By creating and sharing these data we will advance the scientific communities understanding of brain development. In the long-term this can help researchers, clinicians and drug companies develop novel therapies for brain disorders.

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

The short-term beneficiaries of the work will be academics researchers working in this field of neuroscience.

In the medium-term, it is to be expected that this research will provide key insights to those aiming to develop therapies for neurodevelopmental disorders, such as clinicians and pharmaceutical companies.

In the long-term the work is expected to contribute to the development of interventions to improve patient outcomes.

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

We will actively collaborate with others, including existing collaborations and the new ones we hope to develop over the course of the project.

We will present data at conferences, deposit all publications on free websites such as preprint servers and my lab website. We will aim to publish both significant and non-significant findings, as this is important to prevent unnecessary replication of previously performed experiments, thereby helping reduce animal usage.

We will also undertake public engagement work, such as hosting a lab stall at local outreach events.

Species and numbers of animals expected to be used

- Mice: 8000

Predicted harms

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

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

The study involves brain structures that only emerged in recent mammalian evolution and therefore lower species are unsuitable. Mice have been selected as they are the least sentient laboratory species suitable for the study and offer a wide range of transgenic tools to study brain function. These tools are critical for the aims of this study.

The main focus of the study is on brain development during adolescence, due to known changes in brain structure and function occurring at this time and clear links between adolescence and mental health risk. This necessitates studies across the lifespan, from birth to adulthood.

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

Mice will be bred with genetic alterations that enable developmental changes occurring within the brain to be scrutinised. The majority of these genetic alterations (>75%) are not expected to have any welfare consequences for the animals however, in order to investigate the development of neurological disease it is necessary to breed some animals (<25%) with genetic alterations predisposing to the development of clinical neurological

condition. Mice bred with alterations predisposing to neurological disease may develop poor coordination and balance. These animals will be carefully monitored and will be supported by providing food and water at floor level. In the event that their bodyweight drops below 15%, relative to standardised age and sex match control animals, the animals will be killed.

The majority of the animals bred will undergo a surgical procedure, following the induction and maintenance of anaesthesia, to inject substances into the brain to enable developmental changes to be determined (~75%) or to implant an optical probe or, recording electrodes and a head fixation device (~25%). All animals will be given pain killers following surgery and are expected to make an uneventful recovery and to resume normal behaviour within a few hours.

Animals may subsequently be given drugs to modulate neuronal function either via a surgically implanted delivery device or by injection. Animals undergoing surgery to implant a delivery device will be given pain killers and are expected to make an uneventful recovery and to resume normal behaviour within a few hours.

Approximately 25% of the animals will be used in non-aversive behavioural tests. Animals fitted with a head fixation device will be habituated to head fixation and used in reward based tasks. To motivate the animals to take part in these tasks, the animals will be maintained on restricted water access prior to, and during testing.

A small subset of mice (<1%) will receive repeated mild foot electric shocks (maximum of 10). These mice will not have undergone any previous procedures and the effects of the foot shocks are expected to be mild and transient.

At the end of the study, all animals will be killed to enable brain tissue to be harvested for analysis.

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

The majority of genetic altered mice (>75%) are not expected to experience any welfare issues as a result of the modification,

Animals bred with modifications predisposing to the development of neurological disease may develop movement problems or heightened levels of anxiety or over-grooming. These animals will be carefully monitored and will be supported by providing food and water at floor level. In the event that their bodyweight drops below 15%, relative to standardised age and sex match control animals, the animals will be killed.

Mice undergoing surgical procedures under general anaesthesia to implant devices under the skin or into the brain, are expected to experience some pain upon recovery. As far as possible, this will be mitigated by giving them pain killers, which will be maintained until the

animals are showing no further signs of pain. All animals are expected to make an uneventful recovery from surgery and to resume normal behaviour within a few hours.

Some mice will receive repeated injections of certain innocuous drugs to control neuronal function. These injections do not cause more than mild transient pain and animals resume normal behaviour within a few minutes of being given the injection.

Some mice will experience thirst as a result of being kept on water restriction to motivate them to perform behavioural tasks involving a water reward. Water restriction will be titrated to the minimum levels needed to ensure engagement with the task and the animals will be monitored to ensure they retain weight and that their wellbeing is not adversely affected.

Some stress will be associated with behavioural testing and head restraint as mice are placed into unfamiliar environments. This will be reduced by repeated habituation steps that will occur in a multi- step manner. This will ensure the mouse is habituated to handling, movement out of the home-cage environment, habituation to the behavioural apparatus and then finally to head-fixation or tethering to the behavioural apparatus itself.

A subset of mice will receive repeated mild electric foot shocks (maximum of 10). These mice will not have undergone previous procedures and the effects are expected to be mild and transient. Alternative aversive stimuli cannot be used due to the need for them to be highly time-locked, applied to freely- moving mice, and perceived solely by the teacher mouse and not the observer. We will carefully titrate the shock intensity to ensure we use the minimum level to elicit the desired physiological response.

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

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

12% subthreshold

2% mild

86% moderate

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

- Killed

Replacement

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

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

This study focuses on understanding complex developmental changes that depend on diverse in vivo processes, such as hormonal signalling, transcriptional regulation and interactions between different cell types located across multiple brain areas. They can therefore not be studied in reduced systems such as in vitro cultures as these complex interactions are lost when removing neurons from the intact brain. Similarly, they cannot yet be studied in silico as there is insufficient understanding of the biological mechanisms involved to generate computational models.

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

I have undertaken a literature searches and applied knowledge attained during my career as a developmental neurobiologist, including conference and workshop attendance, to explore possible alternatives. I have considered brain cultures, including organoids, computational models, and less sentient model organisms as alternatives.

I am actively collaborating with computational neuroscientists aiming to understand similar circuits to those under investigation in this study.

Why were they not suitable?

Alternatives are not suitable as it is not possible to model the complex developmental cascades that occur in the developing brain in these reduced systems. Neither is there sufficient understanding to build computational models of the brain at the level of detail required for this study. Non-vertebrate animals do not have the same brain regions that we are exploring in this study. Organoids and cell cultures are beneficial for studying early stages of development but cannot develop beyond early gestational periods and so are not suitable for studying later stages 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?

The estimates is based on previous studies I have performed, calculations of necessary breeding colony size and typical litter size and frequency, and consultation with other laboratories performing similar studies.

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

Many of the experiments are designed such that control and treatment results can be measured from the same animal. This greatly reduces the number of experimental mice

needed. Experimental group sizes with be determined using power analyses to ensure adequate statistical power to assess all of the study aims. For behavioural experiments the same mice may be used to test different behaviours reducing the number of mice required.

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

My institute operates a tissue sharing network that enables researchers to provide surplus tissue to others and I will use this scheme to obtain tissues to provide control data wherever possible.

We actively collaborate with computational neuroscientists and will feed the experimental data we generate, over the course of this project, into computational models to improve their predictive power. However, at this stage we do not know enough about the development of brain circuits for the models to provide an accurate picture of what is happening, although they may provide some clues which we can use to guide experimental design and thus reduce 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.

The use of transgenic animals allows us to manipulate cell types within the brain, either to alter the expression and function of endogenous genes or to express exogenous genes that are essential for studying brain circuitry. Transgenic mice are the least invasive way to achieve this, as they do not require surgical procedures (for example: viral injections, or in utero electroporation) to manipulate the genome.

For studies requiring gene expression to be restricted to certain brain regions, or periods of development, it will be necessary to injection viral vectors directly into the brain. Since these experiments require the expression of the gene over protracted periods, it is necessary for the animals to be recovered following surgery. In all cases, surgery is performed under general anaesthesia and all animals are given pain killers upon recovery until no overt signs of pain are detectable. All animals are expected to make an uneventful recovery from surgery and to resume normal behaviour within a few hours.

Behavioural testing allows the functional impact of changes in brain circuitry to be determined and is essential to linking changes occurring at the circuit level with underlying

disease symptomatology. To refine these procedures, we will apply protracted habituation stages and carefully titrate the levels of different stimuli provided to the mice to limit any stressful adverse effects. This includes accounting for phenotype-specific differences across our different mouse strains.

For, behavioural testing requiring head fixation and fluid restriction we will apply best practise, as outlined in a recent NC3Rs working group document (Barkus et al., J Neurosci. Methods, 2022). These will include gradual habituation to handling, the experimental apparatus and head fixation, as well as limiting fluid restriction to early phases of training, where experimentally feasible.

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

Mice are the lowest species that can be used for these studies as the brain circuits under investigation have only evolved in recent evolutionary time, and so are not present in non-mammalian species, for example fish or worms.

We cannot study them at a more immature life stage, as the purpose of the experiments is to observe changes in postnatal development, particularly the period of adolescence. Mice must therefore reach adulthood to meet these objectives.

Behavioural tests are essential to the study as they enable the functional impact of changes in brain circuitry to be determined and linked to the disease symptomatology. It is not possible to conducted these under terminal anaesthesia.

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

Where possible we will use slow-release delivery systems to administer drugs throughout adolescence. This reduces the need for repeated daily handling and injection. Repeated daily injections over long periods (>10 days) will only be used as a last resort if other options are incapable of meeting the experimental aims.

For both water restriction and social learning experiments we will carefully titrate the magnitude of applied manipulations to ensure we evoke the minimal adverse physiological affect while still meeting our experimental objectives. For water restriction steps this will include weaning mice off water restriction as behavioural performance improves with task learning.

We will provide habituation steps for behavioural analysis to reduce stress and improve task performance.

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

I will consult resources provided by NC3Rs, EURL ECVAM and Norecopa PREPARE.

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

I actively engage in my institute 3Rs initiatives, which include workshop, meetings and prizes, in addition to attending scientific conferences and reading NC3Rs literature,

To implement these advances I will consult with experts on the different techniques, at my institute and other resources, for example those provided by NC3Rs, EURL ECVAM and Norecopa PREPARE.

21. New treatments for inflammatory and chronic skin diseases

Project duration

5 years 0 months

Project purpose

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

Key words

vaccine, biologics, inflammatory diseases, therapy

Animal types	Life stages
Mice	adult

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim of this project is to let us look at how safe and effective a new type of treatment might be for long term inflammatory skin disease, such as eczema and psoriasis. The potential treatments we are investigating are substances that are made from a living organism or its products (examples of these types of substances are vaccines and antibodies).

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

Why is it important to undertake this work?

Currently available biologic drugs are successful in many areas of medicine. However, they have important limitations. These drugs often stop working when a patient's immune system starts to produce antibodies against the drug itself. An antibody is a protein produced by the body's immune system to "fight" what is sees as a potentially harmful substance. They also require frequent dosing, and they are extremely costly, providing a massive burden on health care providers and patients. Our new class of active biologic drugs have the potential to overcome these limitations. Prior to trials in people they must be evaluated to check that they work the way we expect and that they are safe.

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

We aim to produce several potential substances that will be suitable to move to clinical trials in people. We will publish the information we gain from our studies so other researchers to benefit from the knowledge we gain around these types of treatment. This knowledge will be potentially useful in many areas of medical research, as similar treatments are being investigated for many types of disease.

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

The information we obtain should allow the initiation of translational clinical trials. These may be trials in either people or animals (for veterinary applications). Potential new treatments are then initially used in small numbers of healthy volunteers and then in people/ animals affected by the disease to check that it can be used more widely. We plan to be able to start such trials for at least one candidate product (potential treatment) within the lifetime of this project.

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

The output will be maximised by liaising with pharmaceutical companies to facilitate clinical translation. Further outputs will be sought by collaboration with other researchers who may adopt our new technology to be applied to other areas of translational medicine. In addition, publication of the technology, so other researchers have access to the information we have gained, will be sought as well.

Species and numbers of animals expected to be used

- Mice: 1500

Predicted harms

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

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

The choice of animals and life stages is selected based on the intended type of studies. The candidate prototype drugs that we aim to evaluate work like vaccines. Once injected,

they stimulate the body to make protective antibodies against a harmful substance, for example a harmful protein that causes itchy skin or inflammation. Non-clinical studies in mice are the most effective model to verify that the candidate drugs indeed are able to stimulate the desired antibody response and that they do not cause any major unintended harm.

An immune response to a vaccine-type drug is best elicited in mice that have reached a certain age (at least 8 weeks) so that their immune system has developed to an adult stage.

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

In general, the type of studies we plan to perform fall into two categories:

a) Testing if the candidate drugs elicit protective antibodies against the harmful protein. For this type of study, the drugs are injected like a vaccine under the skin and the emergence of protective antibodies is tested in blood samples taken at various time points. This type of study may be short-term (up to 8 weeks) to assess generation of antibody response or long-term (up to one year) to assess how long the benefit of the drug lasts, i.e., how long protective antibodies against a harmful substance persist in the body. The duration of this response will give us an idea as to how often we might need to give the treatment to people. How often we need to blood sample is guided by the specific scientific question we are asking, but usually this will only be up to about twelve over the whole year.

b) Test of the how well the antibodies generated against the harmful protein actually work, that is, if they can protect against the disease to be treated. For this type of study, we employ models of human diseases. For example, we will expose the mice to compounds that are known to cause itchy skin or a dermatitis to see if animals which have had prior vaccination with the candidate drug, are protected from this effect. The compounds causing such effects could be injected under the skin or applied as a liquid or cream onto the skin of mice. In this type of study we would also take blood samples to ensure that the mice actually have measurable antibodies before exposing them to a disease-model.

Each of the models we used has been well validated for each disease condition under study and have the least possible harms to the animal whilst letting us find out the answers we need.

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

Blood sampling shouldn’t cause any harm to the mice other than brief discomfort when the sample is taken (like if you have a blood sample taken). We don’t restrain the animals when we blood sample them to reduce any stress.

Subcutaneous injection of active biologic treatments is akin to vaccination of people and so is expected to cause the level of pain you would have if you received an immunisation. Sometimes, some swelling can occur at the site of injection. This is caused by the reaction of the body to the substance used in vaccines to help the body to respond well to the vaccine so the body will build up enough antibodies to be effective. This type of substance is called an adjuvant. We use one called “alum” and it is the same on that is used in vaccines used routinely in people. The response can lead to a small nodule under the skin. In our experience over many years, the animals don’t seem at all bothered by these nodules, but we monitor them carefully and animals would be humanely killed if they seem very uncomfortable or upset due to the lump.

Procedure: subcutaneous injection of cytokines (proteins in the body that help control inflammation). Again, the injection itself is not expected to cause more than brief discomfort. Some of the cytokines we use help us to mimic human skin diseases so are expected to cause itchiness (like people would get with eczema). We measure this by how often they scratch compared to how much they scratched before they were given the cytokine. We expect about a 5-fold increase in scratching for the cytokines that cause itchiness but no other effects on the animal’s welfare. Animals will be given no more than 3 injections of cytokines and only one of the cytokines that cause itchiness. If our treatment works, the itchiness will be less.

Procedure: applying a cream to one ear to cause dermatitis or psoriasis like disease on the ear(s). This procedure is expected to cause a mild-to-moderate dermatitis or psoriasis (depending on which cream we use) localised to the ear receiving dosing. We have a lot of experience of using this cream from previous studies and only use the cream for up to 20 days, often less. Our experience is that the overall well-being of animals is unaffected.

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

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

The expected severity is best described for each procedure in turn:

Blood sampling: mild, proportion: all animals.

Injection of active biologic drugs: mild, proportion: all animals.

Injection of cytokines: mild for most cytokines; moderate for the cytokines causing itchiness: proportion: which cytokines we use will depend on the treatments we need to test. Most animals used on in the project will receive cytokines.

Application of cream to ear: moderate (localised dermatitis or psoriasis on ear for no more than 20 days for animals that have cream that causes dermatitis or psoriasis applied will get some skin inflammation. Below threshold: for animals that only get a cream with no

inflammatory ingredients as these won't have an inflammatory reaction. Only some animals used in the project will have cream applied to one of their ears (around half).

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

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 safety regulations that are in place for human medicines mean that we are obliged to show in animals that the potential treatment is safe and likely to be effective, based on in-vivo data obtained in rodent models before we are allowed to trial the treatment in people. The requirements are defined by authorities such as FDA (US Food and Drugs Administration), MHRA (The Medicines and Healthcare products Regulatory Agency in the UK), EMA (European Medicines Agency).

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

Due to the regulatory requirements detailed above, at this time we cannot use in-vitro / cell culture approaches as no test tube assay can reliably mimic a complex disease. If the regulations change so that non-animal methods are acceptable, we will move to the accepted alternative.

Why were they not suitable?

See above. In addition, for all of the prototype drugs under study, relevant clinical data derived from human clinical trials is used to confirm the clinical effect of blocking the relevant cytokine.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 expected to be used is based on studies conducted under previous licences. The numbers chosen will be the minimum required to obtain a scientifically valid conclusion for each study. The reduction of animals to the absolute

minimum required is guided by experience gathered through similar types of studies conducted under previous licences. The overall number is further reduced by conducting small-scale pilot studies as and when feasible to limit the necessity of statistically powered studies, which require higher numbers.

In general, for each of the candidate drugs, it is expected that a series of prototype variants need to be tested to select the best candidate to take forward. This means that pilot studies using a small number of animals have to be tested to check if the prototype is able to generate effective antibody titres. For selected prototypes further studies will be required to determine the longevity of the antibodies, their effectiveness in a disease model, and if they work in different strains of mice. In some cases, if results obtained from one study turn out to be inconclusive, studies with modified design might be necessary to clarify the scientific question at hand.

Taken together, it is reasonable to expect that for the three different drug candidates planned currently, each will require up to 250 mice.

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

Our approach is that we limit testing of drug candidate prototypes to small short-term (up to eight weeks) pilot studies to confirm if suitable amounts of antibodies can be generated. To further reduce the number of animals, we employ inbred mouse strains where individual mice show a very similar antibody response. This means that the variation is smaller and fewer animals are sufficient to clarify the question at hand.

Once we have established that a candidate prototype drug yields suitable antibody titres, we replace further studies by experiments in the test tube to confirm that these antibodies are able to neutralize the harmful protein which they target in cell culture.

Only once this established, will further studies be designed for selected drug prototypes which have passed the above tests. This means that only very few studies using disease models are conducted with selected candidate prototypes.

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

For each study, previous and pilot studies will help to identify the calculation used to arrive at the required number of animals to be used. For example, we have acquired experience with the disease models described in this licence which enable us to estimate with higher precision the number of animals required to conclusively answer the scientific question at hand, thereby avoiding the need for excessively high numbers of animals, while at the same time avoiding the risk of using too few animals to definitely answer the scientific question.

Refinement

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

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

The animal models used will be the application of either a vitamin-D analogue or a cream licenced for human use (imiquimod) to one ear only. These two models are by far the least invasive of all described in the literature in terms of their impact. They have been outlined above in the section "project harms". By contrast, other models, which have been considered, entail either much longer dosing, or systemic dosing that causes dermatitis or psoriasis that can affect the whole of the body, or use of immunocompromised animals.

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

Mice are the only species where validated models for psoriasis and atopic dermatitis are available. Furthermore, mice are the least sentient species for antibody titre studies informative for clinical development.

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

The procedures will be refined by adopting best practice for the animal environment, thereby reducing stress. Refinement will be achieved by selecting the lowest possible dose of drug based on experience gathered during studies conducted under previous licences.

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

Home Office Code of practice (https://www.gov.uk/government/publications/code-of-practice-for-the- housing-and-care-of-animals-bred-supplied-or-used-for-scientific-purposes)

Best practice methodology in the use of animals for scientific purposes (https://www.nhmrc.gov.au/sites/default/files/documents/attachments/grant%20documents/best- practice-animals-scientific-purposes-2018.pdf)

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

New developments, such as publication of less invasive animal models, will be continuously monitored by review of PubMed database, information shared via our local animal users' group from the NC3Rs and other relevant forums.

22. Trypanosome parasites development and host-tissues invasion

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

African trypanosomes, Sleeping sickness, Quorum-sensing, Nagana, Development

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 are trying to understand how a type of parasites called African trypanosomes, that leads to the “sleeping sickness” disease in humans or “Nagana” in cattle, progresses through its life-cycle. We want to know how these parasites enter different parts of the body and how these changes can affect how serious the disease is.

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

Why is it important to undertake this work?

Trypanosome parasites are part of a larger family called Kinetoplastids, and parasites of this family cause a large range of diseases that have serious social and economic impacts. We want to know what route these parasites use when they enter a new body and to reveal what molecules control these pathways. Finally, we want to understand how the

parasite moves between hosts and between different organs, and how the parasite modifies its own behaviour and functioning mechanisms to adapt to such changes.

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

This research will uncover novel biology of trypanosome parasites by revealing the strategies that the parasites use to ensure their survival inside hosts. In addition, this project will allow us to develop new methods and to use these parasites as models to study infection and immunity more generally in the future.

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

In the short-term, these outputs will be mainly targeted to other scientific researchers and will increase our knowledge of the biology of these parasites. In the longer term, this work may inform ways to combat these parasites which would benefit those who are at risk from trypanosome infection.

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

Data from our animal experiments will be published in open access journals and made freely available in dedicated databases, thereby increasing the exposure and reach to other scientists across the globe. The research will be presented to other members of the scientific community in conference presentations and seminars. Where appropriate, upon publication of the research, a press release will be made to inform the general public of the advance. We will ensure that we publish both positive and negative findings where appropriate.

Species and numbers of animals expected to be used

- Mice: 2400

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 experiments require large amounts of very pure material from living parasites. This material cannot be generated in vitro (i.e. without using living animal hosts) and the best yields come from rodent infections. By obtaining large numbers of uniform parasite populations from a single rodent infection, our experimental analyses are made more robust. A critical part of our experiments is to genetically modify the parasites to understand the role of individual genes. These analyses can only be confirmed by in vivo infections in adult rodents.

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

Typically, adult mice are injected with normal parasites or parasites genetically modified to study particular molecules or genes. Two main types of studies are performed, either following the early phase of infection (up to 20 days), or the later development of the diseases (up to 50 days). Mice can be directly injected with parasites or exposed to the bite of infected tsetse flies to study the complete life cycle. Parasite development is followed regularly by taking small amounts of blood. Both early and late infection studies may require us to measure where the parasites are in the living host and how many parasites there are. To do this, we can use an advanced form of imaging where a chemical agent is given to the infected animals that reacts with a gene in the parasite to emit light. This enables us to see parasites in living animals. The administration of the chemical is done by injection and will be repeated several times during the course of the infection.

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

For most procedures (>70%), we expect that the animals will experience no more than mild, transient discomfort during the procedures. In some cases (<30%) we may observe complications which could include irritation at the site of injection, and stress symptoms from infections. In the latter case, this is estimated to last for no longer than a few hours.

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

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

Mild severity - over 70% of animals. Moderate severity - less than 30% of animals.

We do not expect that any procedure will exceed the moderate category.

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

- Killed

Replacement

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

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

We need to use animals to reproduce the natural life cycle of the parasites and to understand how they are changing during the development of the disease in the blood of the animal.

Moreover, animals are required to investigate how parasites move from the blood to different organs and to understand the role of these organs for the transmission of the parasites

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

Parasites can be grown in flasks in the laboratory, and these ‘in vitro’ culture methods have been developed to study developmental biology of African trypanosomes. To analyse the role of genes of interest for the control of life stage progression, we will edit or remove genes from the parasites before infection. Before infecting an animal, the effect of these genetic manipulations can be tested in in vitro experiments.

Why were they not suitable?

The in vitro environment is vastly simpler than that present in a living animal and in vitro experiments can therefore only provide hints as to the effect of genetic manipulations. Confirmation of these in vitro results in living organisms is an absolute necessity. In addition, parasites obtained from in vitro culture are different from those that grow in animals. This can influence the way they grow, and change their response to drugs. Furthermore, although in vitro tools are valuable, they have the following drawbacks: 1) The parasites are present in low density and 2) parasite differentiation is variable in vitro while parasites in living organisms all derive from a single infection and are therefore homogenous.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 estimates of animal usage are based on consideration of the scope and duration of funded work, staff workload, balance of experimental and analysis time and the need to ensure adequate training and research planning.

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

We have used and will continue to use statistics and the Experiment Design Assistant from the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3R) to design experiments such that the minimum number of animals is used to obtain the maximum information. We will use in vivo imaging to allow longitudinal assessment of parasite infection in an animal. This uses the very latest technologies to

quantify the number of parasites in individual animals accurately over time without killing the animals, consequently leading to a reduction in the number of animals used. Where a new host-parasite combination or a new parasite strain is being used, we will use pilot experiments to establish the inter-individual variability of the infection and attempt to reduce this so that smaller group sizes can be used. Finally, the prioritisation of parasite candidates in vitro will ensure the reduction in the number of studies that are required to be carried out in vivo in animals.

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

We will ensure that pilot studies are performed to refine infection protocols to reduce the variability between animals within a cohort. This typically can include selecting the most appropriate route or dose of infection.

Refinement

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

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

Protocols and routes of infection in mice are well established and described in our research community and the literature. In addition, we have many years of experience of monitoring trypanosome infections in mice and so are able to identify the likely progression of parasitaemia and associated clinical signs. By accurate projection of the course of infection, monitoring can be increased as necessary during critical phases of infection. The predictability of infections in mice also aids the selection of appropriate time points to capture parasites at different stages of differentiation. It minimises the possibility of unanticipated infection pathology ensuring I) that experiments derive maximum outputs from the minimal number of animals used, and II) that no unexpected adverse effect arises, hence avoiding unnecessary pain and suffering for the animals.

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

We require the use of a mammalian species which contains an immune system that has clear similarities with natural host species such as rodents, humans, and livestock animals. The circulating bloodstream and organs in mice present the required molecular interaction enabling the full life cycle development of the parasite. We must use adult mice that have a fully developed and functioning immune system.

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

Procedures will be refined by continual improvement and discussions with scientists, the veterinarian and animal care staff to maintain a culture where the welfare of the animals is central to experimental design. Regular training of all individuals working under this licence as well as regular protocol reviews will ensure that refinements will be implemented promptly.

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

The NC3R's website provides a resource of up-to-date protocols, publications, guidelines, videos and other information that enables the rapid dissemination of the most up-to-date best practices.

Furthermore, we plan our experiments following the PREPARE guidelines and we follow ARRIVE guidelines for reporting of experimental results.

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

We will regularly discuss refinements for animal experiments with the veterinarian and animal care staff. We will keep abreast of developments discussed and implemented by the NC3R through their monthly newsletters and blogs. The animal facility holds regular forums and updates on the current best practice and latest developments.

23. Neuronal circuits of cortical plasticity

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

cortex, vision, plasticity, neuron, autism

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?

The objective of this proposal is to understand how neuronal circuits involved in visual perception are modified by experience. We will determine how behavioral experience modifies the activity of specific neuronal circuits across multiple brain regions involved in integrating visual inputs with other internal and external contextual information.

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

Why is it important to undertake this work?

The results of this project will increase our general knowledge about how the brain stores information and adapts to new environments. In addition to this fundamental knowledge, these results have direct impact into research about pathologies affecting the brain. Knowing how brain functions can be modified by experience will guide strategies to promote recovery of function after brain injuries such as strokes or brain trauma.

It will also facilitate the development and integration of efficient visual aids and hearing aids. With an ageing population, there is an increasing need for visual and hearing aids: an efficient integration of these devices requires functional sensory brain areas that can process this information. Increasing the capabilities of these brain areas to adapt to new stimuli should enhance sensory perception.

In this project, we will also elucidate potential specific defects in the cortical circuits of mouse models of autistic spectrum disorders and intellectual disabilities. These results will give insights into whether and how targeted drugs to specific neuronal sub-populations would be of therapeutic value in these disorders. Finally, these results will be used as a reference for testing how proposed pharmacological treatments can rescue cortical activity deficits in these brain disorders.

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

The primary outputs of the project will be publications of the results in high-impact peer-reviewed journals (published in open access) as well as presentations at conferences and workshops.

This project will advance research into methodologies for the analysis of recordings from very large neural populations, which will benefit neuroscientists using the latest large-scale recording technologies. We will ensure that methodological developments arising from this project will be available to the community by ensuring free access to all data as well as codes used for data processing and analysis. I will ensure the dissemination of this information at relevant conferences and through reviews published in specialized journals.

I have been regularly contributing to public outreach activities through presentations at exhibitions and public engagement events, and I will pursue these activities in the coming years. Two-photon imaging creates beautiful images and videos of neurons, which I am using during public engagement activities and publicity materials such as websites and press releases.

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

In the short-term, the main beneficiaries of this project will be the academic community of neuroscientists.

This project aligns well with the funders challenge area: ‘bioscience for an integrated understanding of health’. Specifically, this project closely aligns with the funders current Key challenge area, Lifelong health and the Strategic priority, Healthy ageing across the life course as there are a number of psychiatric diseases and neurodevelopmental disorders that have been associated with pathological representations of sensory information.

Our results will be highly relevant and timely for the following academic communities:

- The exponentially growing community of neuroscientists studying the neuronal correlates of sensory- motor actions and decision making: (1) they will be able to apply the new tools developed in this project to the analysis of their specific behavioural paradigm and (2) the neuronal representation and plasticity of visual areas will open new avenues for investigating the downstream brain areas processing this information and for studying how this representation is altered in brain disorders, such as schizophrenia or autism spectrum disorder.

- In this project, we will elucidate potential specific defects in the cortical circuits of mouse models of autistic spectrum disorders and intellectual disabilities. These results will give insights into whether and how targeted drugs to specific neuronal sub-populations would be of therapeutic value in these disorders. Finally, these results will be used as a reference for testing how proposed pharmacological treatments can rescue cortical activity deficits in these brain disorders.

- In the field of computational neuroscience, there has been an increased theoretical and experimental exploration of how visual information is represented in the brain. Our study will further this work by providing mechanistic insight into how cortical networks implement a representation of sensory information, which can then be used to inform cortical models.

- This project will also be highly relevant for a growing community of Artificial Intelligence researchers developing artificial agents. While major advances have been made recently in AI, it remains a major challenge to achieve the level of flexible generalisation while remaining robust to several sources of uncertainty, a characteristic of biological intelligence. Understanding how the brain represents sensory information and uncertain contextual information may thus contribute to successfully address this challenge.

- The results of this project will increase our general knowledge about how the brain stores information and adapts to new environments. In addition to this fundamental knowledge, these results have direct impact into research about pathologies affecting the brain. Knowing how brain functions can be modified by experience will guide strategies to promote recovery of function after brain injuries such as strokes or brain trauma.

- It will also facilitate the development and integration of efficient visual aids and hearing aids. With an ageing population, there is an increasing need for visual and hearing aids: an efficient integration of these devices requires functional sensory brain areas that can process this information. Increasing the capabilities of these brain areas to adapt to new stimuli should enhance sensory perception.

This project will create links across disciplines to enable networking between neurophysiologists and computational researchers as well as training of young researchers in the methods from both fields. As such, this project will train a cohort of researchers who will have the complementary skills and expertise to advance the shared interests of both fields.

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

The primary outputs of the project will be publications of the results in high-impact peer-reviewed journals (published in open access). The results from this work will be shared at international conferences for basic scientists and neurologists. We will also engage in public engagement through charities and social media.

I will ensure the publication of negative results and unsuccessful approaches.

Since my appointment as PI in 2013, I have established interdisciplinary collaborations both externally and within the University. I have applied my knowledge in in vivo imaging to other research fields: (1) in developmental Biology (2) in optics and imaging tools (3) in metabolism (4) in computational neuroscience .

Species and numbers of animals expected to be used

- Mice: I will use approximately 6500 mice over 5 years (including about 2500 for 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.

The experiments in this proposal are designed to improve our understanding of synaptic plasticity and neuronal circuit function in the visual cortex of the mammalian brain. In order to record neuronal activity evoked by visual stimulation, these experiments require studying intact neuronal circuits in living animals. It is therefore impossible to avoid the use of animals for addressing these questions.

Mice are the most appropriate animals for these experiments because:

- Basic mechanisms of synaptic plasticity and neuronal circuit functions are likely to be preserved in all mammals including humans.

- Essential knowledge has been accumulated over years of research about the anatomy, the physiology and the plasticity mechanisms in the visual cortex of this species.

- State-of-the-art imaging techniques allowing recordings of neuronal activity in the living brain have also been developed in mice and will be used in this study.

- Transgenic mice offer the unique possibility to study specific neuronal populations, such as inhibitory neurons, in the mammalian living brain. Since this proposal aims at investigating the role of specific classes of interneurons these transgenic mice are highly valuable and necessary for this project.

- Mice have emerged as valuable models of human genetic disorders, offering the opportunity to understand how brain circuits can be altered in genetic disorders and, hopefully, lead to ways in which these disorders could be treated. I will use mouse models of autism spectrum disorders in order to understand how neuronal circuit functions are altered by these diseases.

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

A typical experiment will include:

-Surgery and placement of a chronic recording chamber (head plate) on the skull. This step will be carried out on animals administered a suitable general anaesthetic and mounted in a stereotaxic frame or nose clamp.

- Then, a craniotomy will be performed in the central part of the head plate. The craniotomy will be covered with a sealant (e.g. mineral oil, Vaseline, Quick-fix rubber cap) to keep free of debris before the animal is placed back into the home cage to recover from the anaesthesia.

Animals will be monitored for recovery after anaesthesia before being placed into the home cage and left to recover for a minimum of 24 hours. Post-operative analgesics will be administered as per local Guidelines.

- Viruses and / or marker molecules will be injected into targeted regions of the CNS. This step can be performed either independently or at the same time as the craniotomy. In the latter case, injection of viruses will be performed just after the craniotomy. Then, the craniotomy will be covered with a sealant before the animal is placed back into the home cage to recover from the anaesthesia.

- Habituation and Behavioural training in the head restraint. Animals will undergo 1 training session per day after becoming habituated to the head restraint. Behavioural training will consist of either passively watch visual stimuli or to associate sensory stimuli with a positive reward for performing a voluntary movement (e.g. presentation of fruit juice). Each behavioural training session will last for approximately 60 min, during 4-6 days for simple association tasks and 3-8 weeks for tasks involving a voluntary movement. Animals might be motivated with mildly aversive stimuli (e.g. water restriction-a minimum of 1ml/day for up to 10 weeks or food restriction for a maximum of 15% loss of body weight).

-two-photon Imaging and Electrophysiological recordings. The animal will be head-fixed under a microscope. Two-photon imaging of the exposed brain areas (in the craniotomy performed in step 1) will then be performed. In some cases, electrodes will be inserted into the brain using the same craniotomy, in order to stimulate or record electrical activity. Recording sessions may be carried out to examine long-term plasticity. This may involve multiple recordings from the same craniotomy over the course of 12 weeks, with a maximum of one session per day.

At the end of the experiment, animals will be humanely killed.

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

The highest severity will be moderate. The experiments proposed in this study require surgery, for stereotaxic delivery of viruses to specific brain regions and for the placement of a recording chamber on the skull of the animal. For these experiments, pain will be controlled during surgery by general anaesthesia and post-surgery by analgesics. Deaths resulting from anaesthesia or surgical complications are uncommon (<1%) and will be minimised by correct dosing of anaesthetics, by accurate weighing and by maintenance of body temperature during and post-surgery e.g. use of heat pads. Risk of infection will be minimised by good surgical and aseptic techniques. At the end of each protocol, animals will be killed by using approved humane methods and tissues from these animals may be analysed. Training mice relies on motivating mice to perform a task.

For chronic recordings, prior to training (1-2 days after the surgery) animals will be handled extensively to become familiarised with the surroundings of the recording area and to the experimenter. After 24-48 hrs of recovery, animals will be securely fixed to a head restraint assembly. Animals trained on the cylindrical treadmill will be free to run, walk, groom or remain motionless thus emulating natural mouse movements – this significantly reduces the habituation time to ~1 hr.

Training mice relies on motivating mice to perform a task. Recent studies have demonstrated that water restriction is an effective approach for motivating mice to perform a task. A series of pilot experiments have been performed using water restriction (for a maximum of 20% loss of body weight, animals usually lose 10-15% weight loss and then stabilise their body weight at 85-90%) and were successful . Water deprivation protocol will not start before at least 48 h after any surgical procedure (see protocol 2). We will aim to group house mice, usually in pairs (except for husbandry purposes- e.g. aggressive behaviour where single housing may be used). The water bottle will be removed from the cage of the animals. After 14 h, animals will be provided with 1 ml of water, and then after 24h, again 1ml of water. Pilot experiments show that animals will lose 10-15% of their bodyweight during this initial period of deprivation. Providing a minimum of 1 ml/day afterwards will stabilise the bodyweight between 85-90 %. Water will be supplied to the individual animal in a separate cage for a few minutes.

The health of all mice undergoing water restriction will be monitored and scored daily. Health assessment table and flow chart are provided in the section 'Refinement' and in protocol 2 'Animal experience'. demonstrated that health scores stayed within normal ranges when mice were continuously water-deprived at this level for 3 months. Water deprivation will not be carried on longer than 12 weeks for this project. One project will address long-term consolidation (or forgetting) of memories. For this, mice will be initially trained for a simple task for 5-7 days and then trained to a more challenging task (for example with two different distances and visual cues associated with the reward, in the

virtual reality) for 2-3 weeks. On the 5th and 6th weeks, we will test whether the mice remembered the initial task while still performing the more challenging ones. Depending on the results, we may add a third level of difficulty with the reward associated with a given probability (one visual cue would be associated with a high probability of reward while another one will be associated with a low probability). Animals will be trained for 2 additional weeks and finally tested again to assess whether they remembered the initial task.

Behavioural training will only start once the animals have become fully habituated to being head restrained. This will be achieved by placing head-restrained animals on a friction reduced treadmill where animals can choose to remain still or walk freely. Two-photon imaging should not cause adverse effects. Animals will be closely monitored while undergoing imaging. Behavioural training will be conducted in the animal facility where noise will be kept to a minimum to avoid unnecessary stress. All experiments will be conducted in a dedicated room. Two-photon imaging should not cause adverse effects. Animals will be closely monitored while undergoing neuronal activity recordings. Any deviation from normal health will result in being monitored more frequently. Monitoring will be according to local guidelines.

For Aim 2 (determine the effects of calory intake on neuronal function in V1), mice will be food restricted. Animals will have a minimum of 72 hours to recover from surgery before commencing food deprivation and behavioral training. Animals will be food restricted to 85-90% of their reference body weight, which will be calculated based on pre-experimental baseline weight and standard growth curves . Animals will have access to a daily ration of food depending on their baseline weight.

In addition to daily monitoring of body weight, a deprivation score will be calculated for each animal. Health assessment table and flow chart are provided in the section 'Refinement' and in protocol 2 'Animal experience'.

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

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

for animals involved in experimental procedures: 95% of mice will be of moderate severity, with 5% being mild.

for animals used for breeding: 100% mild

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

- Killed

Replacement

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

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

The experiments in this proposal are designed to improve our understanding of synaptic plasticity and neuronal circuit function in the visual cortex of the mammalian brain. In order to record neuronal activity evoked by visual stimulation, these experiments require studying intact neuronal circuits in living animals. It is therefore impossible to avoid the use of animals for addressing these questions.

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

Use of alternative computational approaches.

In undertaking the proposed programme of work, I will wherever possible take advantage of computational approaches. First, to make predictions that can be used to guide the design of future experiments. Second, to ensure experiments are effective at testing hypotheses and therefore to reduce the probability of unnecessary or unhelpful experiments being carried out. Nevertheless, it is not possible to generate new physiological data from computational models. This can only be obtained by carrying out experiments on suitable animal models.

Why were they not suitable?

Nevertheless, it is not possible to generate new physiological data from computational models. This can only be obtained by carrying out experiments on suitable 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?

I have utilized statistical analyses to estimate how many animals are needed per experiment. These analyses are based on previous experience with this type of experiment and expectations of an expanding number of researchers and projects over the next five 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 will be minimised wherever possible, and animals and brain tissue will be shared across experiments as much as possible.

Chronic recordings (imaging or recording the same neuronal population over consecutive days) performed in the same animal do not only provide essential information about the plasticity of neuronal activity but also allow for the reduction of the number of animals used, compared to acute recordings in which different animals are used and sacrificed at different time points. Furthermore, different data types will be collected from each animal including electrical, video and anatomy, which will generate a large data set with multiple measured variables for each animal.

Experimental work will be complemented with theoretical modelling to further minimise the number of experiments and animal use. The estimates for the number of animals required are based on my past experience and on published literature. Because the project addresses fundamental questions in well controlled experimental set ups, I will be able to adopt a hypothesis driven approach that will maximize the chance of each experiment to produce clearly interpretable results.

I use power measurements to estimate the number of animals required.

The use of state-of-the-art technologies, such as two-photon imaging and patch-clamp electrophysiological methods, that offer high signal-to noise ratio, will contribute to the generation of clearly interpretable data and as such, minimize the number of animals that are required to test a particular hypothesis.

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

Most animals will generate data including several hours of brain activity as well as behavioral parameters such as motor movements and pupil dilation. These large data sets will be analysed in great detail utilizing advanced analyses such as machine learning to optimize the outcomes from these multi-variable, big data sets. The data will be shared publically such that the same data set could be analysed in parallel in different labs testing different (and potentially complementary) hypotheses.

Since I have established my research group, I continuously worked with computational neuroscientists and theoreticians by sharing the data acquired in the lab and by contributing to the development of new analysis tools.

Refinement

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

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

We will use mice as experimental models.

The methods are chronic imaging and recordings of neuronal activity in awake behaving mice performing motor and cognitive tasks.

Pain is controlled during surgery by general anaesthesia and post-surgery by analgesics. Deaths resulting from anaesthesia or surgical complications will be minimised by correct dosing of anaesthetics, by accurate weighing and by maintenance of body temperature during and post-surgery

e.g. use of heat pads.

For chronic recordings, prior to training (1-2 days after the surgery) animals will be handled extensively to become familiarised with the surroundings of the recording area and to the experimenter. Animals trained on the cylindrical treadmill will be free to run, walk, groom or remain motionless thus emulating natural mouse movements.

Behavioural training will only start once the animals have become fully habituated to being head restrained. This will be achieved by placing head-restrained animals on a friction reduced treadmill where animals can choose to remain still or walk freely. Behavioural training will be conducted in the animal facility where noise will be kept to a minimum to avoid unnecessary stress. All experiments will be conducted in a dedicated room. After each training session, animals will be returned to their home cages. They may be maintained in a reversed light/dark cycle to facilitate their well-being by synchronizing their activity with experimental schedules. The health of all mice undergoing behavioural training will be monitored and scored daily. See health assessment table and flowchart in Protocol 2.

Control groups:

For Aim 1 (To determine how neuronal circuit activity is modified during the active learning of a visually- guided reward task). Animals will act as their own controls: comparing activity before and after learning of a task, comparing activity during the execution of a task with activity in total darkness, and comparing activity before and after a particular treatment (such as the activation of an opsin).

For Aim 2 (To determine the effects of calory intake (diet) on synaptic, cellular, and network function in V1). Animals under food restriction will be compared with animals with ad libitum access to food. For some experiments, animals will act as their own controls: comparing activity before and after food restriction.

For Aim 3 (To determine whether visual cortical circuit function is disrupted in the brain of mouse models of intellectual disabilities and autistic spectrum disorders (Fragile X and

Syngap mutation). Experimental groups will be defined by genetic status, modified or control wild-type littermates. Wild type littermate controls will be used as controls.

The experimenter will be blinded to the genotypes (or food restriction state) of the animal throughout the duration of the experiment and in the subsequent analysis. The selection of animals in each group will be determined by a randomization analysis utilizing software generated in the lab.

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

The experiments in this proposal are designed to improve our understanding of synaptic plasticity and neuronal circuit function in the visual cortex of the mammalian brain. In order to record neuronal activity evoked by visual stimulation, these experiments require studying intact neuronal circuits in living awake animals that perceive visual stimuli. It is therefore impossible to avoid the use of animals with a visual system for addressing these questions.

In order to study how sensory information propagates through complex brain networks that are clinically relevant it is necessary to use brains that highly approximate human brain. Rodents have the overall same structure as human brain with a cortical mantel and underlying structures. This cannot be done in less sentient animals that do not have brain structures that allow for higher cognition.

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

We are primarily using technologies to perform long-term recordings in each experimental mouse.

The experiments proposed in this study require surgery, for stereotaxic delivery of viruses to specific brain regions and for the placement of a recording chamber on the skull of the animal. For these experiments, pain will be controlled during surgery by general anaesthesia and peri-operative analgesia. Deaths resulting from anaesthesia or surgical complications are uncommon (<1%) and will be minimised by correct dosing of anaesthetics, by accurate weighing and by maintenance of body temperature during and post-surgery e.g. use of heat pads. Risk of infection will be minimised by good surgical and aseptic techniques. Surgical sites will be monitored for signs of inflammation and infection. Appropriate effective treatment e.g. antibiotics will be administered under the advice of the Named Veterinary Surgeon if required.

For chronic recordings, prior to training (1-2 days after the surgery) animals will be handled extensively to become familiarised with the surroundings of the recording area and to the experimenter. After 24-48 hrs of recovery, animals will be securely fixed to a head restraint assembly. Animals trained on the cylindrical treadmill will be free to run, walk, groom or remain motionless thus emulating natural mouse movements – this significantly reduces the habituation time to ~1 hr.

We will aim to group house mice, usually in pairs (except for husbandry purposes- e.g. aggressive behaviour where single housing may be used).

The health of all mice undergoing water or food restriction will be monitored and scored daily demonstrated that health scores stayed within normal ranges when mice were continuously water deprived at this level for 3 months.

Behavioural training will only start once the animals have become fully habituated to being head restrained. This will be achieved by placing head-restrained animals on a friction reduced treadmill where animals can choose to remain still or walk freely. Two-photon imaging should not cause adverse effects. Animals will be closely monitored while undergoing imaging. Behavioural training will be conducted in the animal facility where noise will be kept to a minimum to avoid unnecessary stress. All experiments will be conducted in a dedicated room.

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

Procedures for Behavioral Experiments in Head-Fixed Mice, Plos One, February 10, 2014.

Refinements to rodent head fixation and fluid/food control for neuroscience, Journal of Neuroscience Methods, Volume 381, 1 November 2022.

The NC3Rs dedicated webpage on refining these models, that provides references and other resources to use throughout the project. See https://www.nc3rs.org.uk/3rs-resources/refining-use- head-fixation-and-fluid-control-rodents

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

I will consult scientific literature on 3Rs, take heed of local announcements of the 3Rs in my institution and follow 3R accounts on social media.

24. Evaluation of therapies for narcolepsy

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

Narcolepsy, Cataplexy, Nervous system dysfunction, Rodent disease models, New therapies

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The overall aim of this project is to develop rodent models of narcolepsy, which will then be used to evaluate the effectiveness of potential therapeutics. This will aid the development of new pharmacological approaches to treat this debilitating condition.

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

Why is it important to undertake this work?

Narcolepsy is a debilitating chronic sleep disorder characterised by excessive daytime sleepiness, sleep fragmentation, dream-like hallucinations and for some patients (narcolepsy type I) episodes of sudden loss of muscle tone (cataplexy). Symptom onset occurs between 7 and 25 years of age with patients experiencing frequent and often multiple daily attacks for the rest of their lives. Narcolepsy is estimated to affect about 1 in 2,500 people meaning approximately 30,000 people are affected in the UK. Narcolepsy is a chronic disease that can affect many aspects of daily life including education (negative impact on academic performance), employment (higher rate of unemployment), driving (increased risk of accidents) and relationships and emotional health (anxiety, depression). The treatment options currently available for narcolepsy are often unsatisfactory due to suboptimal efficacy, side effects (including strong abuse potential, psychosis, depression and cardiovascular effects) and development of drug tolerance. A European survey conducted on narcolepsy patients reported that, despite current treatment, 84% of patients still felt the negative impact of symptoms on a daily basis, strongly suggesting that better therapies are needed for this disease. The attacks cause disability, interfere with normal function, and are life-limiting. Patients often struggle to work full time and become fearful of the attacks and often develop significant anxieties leading to a very poor quality of life and a significant burden on the healthcare systems and carers. This project constitutes part of Vertex's efforts to develop novel therapeutics directly tackling the cause of the disease to reduce excessive daytime sleepiness, restore sleep homeostasis and stop cataplexy attacks hence improving the patient's quality of life with significantly less undesirable side effects.

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

This work is expected to provide:

1) Novel information about the regulation of wakefulness and arousal and an increased understanding of the pathophysiological mechanisms involved in narcolepsy with and without cataplexy.

2) Data on how well potential therapeutic compounds are tolerated and their effectiveness in treating the underlying causes and symptoms of narcolepsy. This 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 trial within the 5-year life span of the license.

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

Testing potential therapeutic compounds in disease models of narcolepsy and cataplexy 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. In addition, data from studies may 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 narcolepsy and cataplexy 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: 12000
- 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.

All the animals used in this project will be rodents. We will initially use a well characterised mouse model of narcolepsy that has been thoroughly described in the literature, but will also investigate two more recently developed mouse models where narcolepsy symptoms can be induced at a specific age. These models more accurately replicate the post pubertal onset of human symptoms. 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 for a species with a mammalian brain. The rat offers a larger brain which can be beneficial for mechanistic electrophysiology studies and is essential for microdialysis work with repeated sampling.

Embryo, neonate, juvenile, pregnant and adult life stages will be used for breeding and maintaining mouse colonies, but most studies will be performed in adult mice. Juvenile rodents will primarily be used for ex-vivo electrophysiology studies because brain slices from this age of animal remain viable for longer. Chronic efficacy studies will be performed in adult animals since they display the full range of narcolepsy symptoms observed in human patients.

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

Genetically altered mouse models of narcolepsy will be bred, and sometimes induced, to display clinical signs of disease.

Mice will be dosed with compounds in order to investigate their ability to restore sleep patterns in narcolepsy models or promote wakefulness in wild-type mice. Compounds will be given orally, by injection, or via surgically implanted cannulas/ minipumps (required when compounds must be administered directly to the brain or a continuous supply of compound is required). This can continue for a period of a few days (for tolerability studies) or up to several weeks (for studies investigating treatment effectiveness).

Mice may also undergo surgery to implant devices that allow monitoring of motor activity or sleep states. Recordings can then take place as animals move freely for up to 90 days.

Some mice will have blood samples and cerebrospinal fluid samples taken to assess markers of disease progression.

A small number of rats will be used for microdialysis studies under terminal anaesthesia.

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

We have worked with the well characterised mouse model of narcolepsy under a previous licence and found that it has narcoleptic episodes but relatively infrequent cataplexy events. The inducible mouse models of narcolepsy are reported to have more pronounced narcoleptic episodes and more frequent cataplexy events. However, these models better replicate the onset of symptoms in human disease and provide a larger window for examining compound efficacy.

Animals are expected to experience momentary pain and stress during administration of therapeutic compounds. Some 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. This should reduce the chances of such effects being seen in larger efficacy studies. 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, are likely to involve body weight loss and deterioration in clinical signs. Any animals exhibiting such signs, which cannot be ameliorated, will be killed promptly and humanely.

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

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

If animals experience the maximum severity permissible under each protocol the proportion of animals in each category would be:

Mice - 25% mild and 75% moderate. Rats - 100% moderate.

However, we do not expect all animals to experience the maximum severity permissible.

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?

Mouse models of narcolepsy are essential to evaluate the effectiveness of test compounds against more complex clinical symptoms such as sleep, wakefulness and cataplexy, which can only be assessed using an intact mammalian brain. Testing in such a 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 trialed. 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 cannot reliably predict in vivo effectiveness of compounds against more complex phenotypes such as sleep, wakefulness and cataplexy. Patient postmortem brain is difficult to source and, similarly to cells, does not fully recapitulate the symptoms observed in narcolepsy 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 number of animals used in the project is based on previous work in this area and is sufficient 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. 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 activity and behaviour). 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 to minimise excess animals 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.

We will employ a re-use protocol for EEG/EMG recording experiments. Animals that have undergone surgery to implant EEG/EMG devices will be dosed with up to 3 separate compounds. This representsa reduction because the efficacy of three compounds can be tested for every EEG/EMG implant surgery performed.

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 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.

The narcolepsy mouse models that we plan to use display a disease phenotype that mirrors the major symptoms of the human condition. They are based on the same mechanism that has been shown to cause both cataplexy and excessive daytime sleepiness in narcolepsy patients, therefore improving the likelihood of translation of efficacy observed in these models to clinical benefit. We will initially work with a well characterised model, which we used under a previous licence, and was found to have narcoleptic episodes but relatively infrequent cataplexy events. We may also employ inducible narcolepsy models, which are reported to have more narcoleptic episodes and an increased frequency of cataplexy events. Although the phenotype of the inducible models is more pronounced, they better replicate the onset of symptoms in human disease and provide a larger window for examining compound efficacy. We also have experience from previous and ongoing projects of developing strategies to mitigate mouse models with harmful phenotypes.

This project uses techniques that can also be used in patients during clinical trials such as detection of sleep-wake cycles using electroencephalogram (EEG) recordings and muscle activity using electromyography (EMG) recordings. These techniques will provide efficacy data on key symptoms but also crucial information on the mechanism of action of the compound tested that will be directly translatable to the clinical situation. The EEG/EMG recording system we will use is wireless and hence requires no tethering cables or headcaps. After EEG/EMG devices have been surgically implanted, all recordings can be performed while animals move freely. In addition, we will trial adding companion animals to recording chambers to mitigate isolation stress. All surgeries will use high standards of aseptic technique as well pre- and post-operative care to minimise pain and aid recovery.

Neurotransmitter levels, which are altered in narcolepsy, will be measured by microdialysis in rats under prolonged anaesthesia. During this time cardio-respiratory function and response to pinching will be regularly monitored. Any indication of a decrease in depth of anaesthesia will be immediately counteracted by increasing the dose of anaesthetic.

Respiratory depression will be counteracted by injection of a respiratory stimulant. Alteration of physiological parameters will be avoided by using a heating blanket, with a temperature probe feedback control, to maintain body temperature. Body fluids will be maintained by injection(s) of saline.

Pilot studies may be conducted in small numbers of animals in order to refine the parameters and methodology used in subsequent larger studies. These pilot studies are intended to define the harm/ benefit ratio of each procedure, with the aim of generating robust data whilst causing the minimum amount of suffering to animals. They may also provide data for power calculations to determine the number of animals required in larger efficacy studies.

Compound tolerability studies will be performed in small numbers of animals to reduce the likelihood of dosing-related adverse effects becoming apparent in larger studies.

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

The mouse narcolepsy models we plan to use replicate the symptoms and underlying mechanism of the human disease, therefore recapitulating the known causal human biology and improving the likelihood of translation from efficacy observed in these models to the clinic. 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. An intact mammalian brain is also important for modeling more complex clinical symptoms such as sleep, wakefulness and cataplexy.

The larger brain of rats is beneficial for mechanistic electrophysiology or essential microdialysis studies with repeated sampling. Terminally anaesthetised rats will be used in some instances where mechanistic rather than behavioural read-outs are required.

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

Regular evaluations of procedures and their associated welfare implications will be conducted throughout the project. Findings will be discussed in the context of improvements that can be made without impacting on scientific outcomes at AWERB meetings and bi-annual licence inspections. We will also seek advice from the NACWO and NVS, as well as other scientists working in the in vivo facility.

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.

25. Anti-cancer therapy validation

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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, Immune cells, Hypoxia, Tumour microenvironment, Therapy

Animal types	Life stages
Mice	adult

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

To generate robust proof-of-concept data that either drives forward or stops progression of 3-5 anti- cancer therapies. We are focussing on therapies that influence the local environment of the tumour (called the tumour microenvironment) and/or immune contexture, which is the number, location and type of immune cells in the tumour or in the bloodstream.

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

Why is it important to undertake this work?

According to Cancer Research UK statistics there are around 375,000 new cancer cases in the UK every year, and around 167,000 cancer deaths. Whilst significant progress has been made in the treatments for many types of cancer, some have seen little improvement over many years. In these latter cancers of current unmet clinical need, standard-of-care treatments are often poorly tolerated, with significant side effects and negative impact on patient quality of life. Using new therapies that are able to target more specific

characteristics of cancer can have significant benefits to patients.

Examples include the use of immunotherapies and molecular targeted agents. However even here, patients can show variable response and over time, the therapies can stop working. It is important to undertake further research and animal studies with new therapies for cancers of unmet clinical need, ensuring that we gain a fuller understanding of how, when and where to use them to maximise patient benefit.

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

This project will generate data, new knowledge in the area of cancer. We will learn more about the interaction between cancer cells, the tumour microenvironment and the immune system, which controls how well a tumour responds to treatment. We will generate proof of concept data i.e. information on the effectiveness and/or side-effects of new therapies. This proof-of-concept is needed to progress a specific therapy to clinical trials. Alternatively, the proof-of concept data may show that the therapy tested is not very effective and/or has unacceptable side effects, which is essential information to enable us to say that particular therapy is a “no-go” and should not proceed to trial in humans. Further, benefits will include publications and presentations, to both scientific and public groups.

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

Over the duration of the project the main benefactors from this research will be the research group, others at our establishment, other researchers and, potentially, pharmaceutical industry should they collaborate in the research programmes undertaken. Over longer timescales (5-10 years), progression of therapeutic approaches into clinical trial would yield benefit to patients and clinicians.

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

Work (with both positive and negative outcome) will be published in open-access journals, presented at scientific meetings, shared with public and patient groups and funding bodies. Resources will be made available to other researchers (e.g., data, animals, tissues) to enable collaborative work. Data, where not prohibited by patent law, will be deposited in publicly available databases such as MGI (mouse genome informatics), MGB (Mouse Genome Biology) and XNAT (imaging data). We have an internal system within our groups in the department and more broadly at the establishment to offer materials that are not being used for our experiments but may be useful for others e.g. we often donate bone marrow from our mice to groups working in that area. I have national and international networks with researchers in the same field and we routinely share our in vivo data outcomes in order to generate consensus guidelines and refine processes moving forward. I have personally been involved in creating these guidelines at national and international level.

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.

This project will use adult mice because it is not yet possible to model fully the complexity of the interacting cell populations within a tumour, the dynamic state of the local environment of the tumour (called the tumour microenvironment) or complex normal tissue responses that may underpin toxicity in any lower species of animal or at any early life stage than adult mice.

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

Animals will be anaesthetised and implanted with identification chips and tumour cells under the skin or in specific sites in the body to match the human disease e.g. brain cancer cells into the brain. Tumour growth will be followed by direct measurements of tumours under the skin (eg using callipers or imaging or if the tumours are in another part of the body and can't be measured by callipers, via imaging) for which animals will be anesthetised and may receive a “tracer” that allows contrast to be seen between the growing tumour and surrounding normal tissue. If required to follow metastatic tumour growth and/or therapy response, surgical tumour removal will be performed. Therapies will be administered via standard injection routes, sometimes in combination with, for example radiotherapy (that is restricted to the tumour site) or standard therapy in the clinic currently e.g. chemotherapy or immunotherapy. Samples (blood, tears or tumour biopsy) may be taken to monitor changes over time. Prior to humane killing at the end of the experiment, animals may receive pathophysiological markers that allow clearer evaluation of tumour and/or normal tissue biology at post mortem. Animal weight, wellbeing and tumour condition will be monitored regularly throughout using refined approaches and appropriate interventions (e.g. dose reduction, decreased frequency of dosing, supportive measures such as mash or topical emollients for skin dryness) made where necessary.

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

Most animals will be implanted with tumours. Tumour implantation and growth may cause some changes to skin condition. Animals will feel transient pain/discomfort upon administration of therapeutic agents. Weight-loss/reduced of weight gain compared with naïve animals may be observed over the time-course of therapy interventions. Late stage disease/pathology may induce change in well-being, condition and behaviour of very short duration as this would indicate termination of the experiment.

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

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

Moderate for >90% of animals, mild for <10%.

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

- Killed

Replacement

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

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

We cannot yet model fully the complexity of the interacting cell populations within a tumour, the dynamic state of the tumour microenvironment or the systemic complexity that may underpin toxicity (e.g. cognitive decline following brain tumour treatment) in any non-animal system (e.g in cells grown in the lab). Ethically we cannot test these facets in the clinical setting and therefore must use species with physiology that best represents what we would expect to find in the human diseased state.

Therefore, we need to use animals (mice) as they very closely model the complexity of whole organ system interactions in human disease. Animal studies will enable us to answer the key aims of this project, as they allow us to determine the holistic effects (ie effects on multiple systems concurrently such as the tumour, the immune system, blood flow, oxygen levels) of therapies, which is ultimately what determines their overall efficacy.

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

However, to replace the use of animals, where possible, we are working with materials scientists and engineers to develop complex systems outside the body/animal as better future mimics, which are informed by what we learn about cancer biology in our animal models.

Before initiating mouse studies we undertake comprehensive studies in tissue/cells grown out of the body, which include using 3D-cell systems and modified culturing conditions, such as manipulation of oxygen availability, to mimic tumour conditions inside the body. Coupled with computerised models that tell us how the drug will interact with the chosen target in the body and predict how the drug will be absorbed into the body and processed so that it reaches the tumour (this is called Pharmacokinetics), these important pieces of data enable us to make informed go/no-go decisions as to whether a study should progress to using mice.

Why were they not suitable?

We routinely use these systems to do thorough testing of potential new treatments and the cancer biology associated with them to be sure we should progress to in vivo work. In some instances, non- animal systems are sufficient to answer basic questions (e.g. do the treatments make tumour cells grow slower or die) on potential new treatments and therefore can partially replace animals.

However, although these non-animal alternative systems are useful for making informed go/no-go decisions as to whether a study should progress to in vivo work, we are still unable to appropriately and reproducibly model the complex, dynamic interplay between the tumour, its local environment and the whole organism - and it is the complex interplay of these factors with the therapy that dictates if the therapy will work.

As a result of these limitations of non-animal models, to achieve the aims of this project studies need to be conducted in whole animals.

Reduction

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

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

The number of mice estimated to be used per annum is based on our previous experience of delivering analogous objectives to those here and the usage from other similar licences e.g. previous PPLs of collaborators and colleagues.

From this, we have a very good estimate of the number of mice it takes to deliver the package of data required by drug regulators to decide if a treatment should progress to clinic - the so called "go/no go decision making" for clinical trial.

For example, a series of experiments to get proof of concept in protocol 1 consists of approximately 60 animals and 25 studies are planned over the license period giving an expected requirement of 1,500 mice.

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

We will use the study design methodologies we have developed over previous licences that reduce the numbers of animals used by which aim to reducing variability, improving experimental consistency and confidence in outcomes. Inherent variability is minimised by using adult, same sex, age matched animals.
The data we generate for individual models is used as a cumulative resource for sample size calculations (ie we continually pool control data or single intervention data where we are interested in the impact of a novel combination approach). Having robust control/single intervention data to use in sample size calculations reduces the number of animals needed to demonstrate an effective outcome of a novel intervention .

To reduce the numbers of animals used in tumour studies, treatments will be initiated when tumours are of equivalent size which we have previously shown offers a substantial reduction compared to using time-matched treatments on tumours of variable size and underlying pathphysiological heterogeneity.

We will use the NC3Rs Experimental Design Assistant for new more complex studies- we have previously adopted this approach in our normal tissue work whereby we are integrating multiple assessments- behaviour, imaging, post-mortem tissue analyses - and which reduces the numbers of mice required to generate statistically meaningful data.

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

Our routine adoption of imaging allows continuous non-invasive monitoring of disease progression, reducing numbers and enabling us to intervene earlier with minimal detrimental effects.

Archived control data (non-treated animals) are used as a cumulative resource to allow

recalculation of sample sizes and consequent reduction in group sizes. If several related agents are assessed, initial experiments use 2 animals per dose level. If both fall beyond the 95% confidence interval for the control data, the agent is taken forward.

Pilot studies will be used, for example if: 1) a model hasn't been established in mice by the group previously, 2) the likelihood of a model spreading to other sites in the body (metastases) has not been established 3) the marker in the mice that we want to use to track the biology of the cancer and/or track the response to treatment has not been previously evaluated (these tracking methods could be imaging or tissue based: blood, tears, tumour biopsy). The numbers per group will not exceed 6 for (1), 12 for (2) [spread to other parts of the body (metastases) is less uniform i.e. more variable than tumour growth, so requires increased numbers] or 6 for (3). Data from these initial pilot experiments will be used to define group sizes using power analysis.

We run monthly "In Vivo User Group" meetings, which provides a forum for us to share tissue from upcoming experiments. Examples of this is providing colons from irradiated mice to a colon research group, harvesting bone marrow for the immune cells for immunology research and harvesting skin for use in skin research labs.

Refinement

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

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

Mice will be used as they are the species with the lowest neurophysiologic sensitivity in which a range of well-characterised models of cancer exist. Further correlative studies between mouse and man indicate the potential for results to translate between the species.

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

Mice of a more immature life stage or less sentient species lack the complex, dynamic interplay between the tumour and the micro and macro environment, particularly with respect to the effects on the immune system: a key readout of the efficacy of the anti-cancer therapies we are investigating.

Therefore, we cannot achieve our aims by modelling cancer in any organism of lower order than adult mice.

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

We have refined and developed imaging approaches that inform tumour size, how their blood vessels are working, how much oxygen they have and, by extrapolation areas of the tumour that are still functioning (viable) and those that have been destroyed by the therapy

(non-viable). Furthermore, data acquired from imaging studies is being used as a resource to develop data-derived mapping of tumours with a goal to stratify regions dependent on response. All of these approaches are fully translatable to the clinic.

We have moved away from using injectable anaesthetics by adapting equipment to allow gaseous anaesthesia use that allows more control of the depth and duration of sedation.

In our surgical techniques, we have moved away from using skin staples and reverted to stitching which enables more freer movement of muscle and skin layers whilst healing which reduces irritation to the animals. We also use a painkiller (eg buprenorphine) before a surgical procedure called a laparotomy, so that it is active by the time the mouse recovers from surgery.

We have found that for some tumour models, scabbing occurred, which can be reduced by implanting the cells at an increased distance away from the injection entry site.

We don’t cull immediately mice that haven’t grown tumours, but instead use them as company for mice remaining on study.

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

Adherence to LASA Guidelines, Workman et al, Joint Working group on refinement (superseding LASA for substance administration), PREPARE guidelines: readily available, fully researched guidelines that provide clear guidance on how to conduct animal experiments such that the harm:benefit ratio of the work is optimal.
We will also follow guidance developed by my group and collaborators groups, during the course of previous licences of which these are some examples:
CRUK Roadmap for developing imaging-based biomarkers

FOSTER guidelines for developing patient-derived models and preparing European wide guidance on the optimum use of osteosarcoma (bone cancer) models in animal research.

Collaborator - whose team will work under this PPL - has developed policy guidelines for the refinement of drug and radiotherapy studies at international level, which has been adopted by drug regulators around the world (FDA, EORTC and AACR).

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

Newsletters from the "NC3Rs" and Establishment "3Rs Managers". "NC3R" is an initiative that funds research into and shares information on the reduction, refinement and replacement of animal research.

"LASA Guidelines" and "Workman et al, Joint Working group on refinement" (superseding LASA for how to administer therapies) and "PREPARE guidelines" are all readily available documents that provide clear guidance on how to conduct animal experiments such that the harm:benefit ratio of the work is optimal.

We have presented data at and attended meetings in these areas and will continue to do so.

We have regular local meetings for all colleagues who work with animals under our PPL.

We use this forum to share best practice and discuss ways we can refine our procedures. We work alongside several other groups locally, nationally and internationally.

26. Can we modify the severity of inherited cardiac conditions? A study on novel treatments and environmental factors that modify the severity of inherited cardiac conditions

Project duration

5 years 0 months

Project purpose

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

Key words

Inherited cardiac conditions, diet, pollution, systemic inflammation, low potassium levels

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

Inherited cardiac conditions (also known as genetic cardiac conditions) are caused by genetic defects that cause abnormalities of electrical activity and/or pumping function of the heart. The overall aim of this licence is to get a better understanding of the mechanisms responsible for the onset genetic cardiac conditions and to develop treatments for these conditions.

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

Why is it important to undertake this work?

Genetic cardiac conditions tend to affect children, adolescents and young adults and can cause significant symptoms. In a subset of patients, inherited cardiac conditions cause cardiac arrest that without prompt resuscitation results in sudden death. Inherited cardiac conditions are the leading cause of sudden death in adolescents and young adults. Over the last decade these conditions have rose to prominence following the cardiac arrests of high-profile footballers on the football pitch. Their cardiac arrests were all caused by genetic cardiac conditions. Currently, there are no treatments that are specific for these conditions. In the majority of cases, we can treat patients to reduce the symptoms, but we are not able to give any treatments to specifically correct the abnormalities responsible for these conditions. In many cases patients need to have a cardiac defibrillator implanted to be protected from sudden death. Within families, there is significant variability in the severity of these conditions. Some family members are badly affected while others have no signs or symptoms of the condition despite carrying the same genetic defects responsible for the condition. There is a desperate need to identify novel treatments specific for these conditions. In addition, there is an urgent need to understand whether factors such as diet, low potassium levels in the blood, inflammation, and exposure to environmental pollution can modify the severity of these conditions. Identification and removal of factors that modify the severity of these conditions could help in their treatment.

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

This project is likely to generate several important outputs that will impact in the management of patients with genetic cardiac conditions, further advance our understanding of the mechanisms responsible for the onset of these conditions and help us understand the factors responsible for the variable occurrence (penetrance) of these conditions.

These will be:

Novel treatments of genetic cardiac conditions re-tasking existing drugs already used in patients for other conditions and novel drugs therapies.

A greater understanding of how genetic cardiac conditions cause heart failure through identifying the mechanisms by which abnormalities in electrical activity and/or pumping function of the heart cause heart failure.

Identify factors that influence penetrance and severity of inherited cardiac conditions. These will include diet, levels of potassium in the blood, levels of inflammation in the body and exposure to pollution.

Our principal output will be via publications in high quality journals, which will inform the work of other scientists around the world.

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

Short term beneficiaries

Research scientists could benefit directly. We will report our findings in peer reviewed publications and presentations at national and international meetings. The study will explore novel therapeutic options for genetic arrhythmia syndrome and directly explore the mechanisms of arrhythmias in lamin A/C cardiomyopathy and mitochondrial dysfunction in hypertrophic cardiomyopathy. These studies in addition to potentially improving the treatment of these conditions will also provide a more detailed understanding of the

mechanisms that are responsible for the onset of these conditions. The studies on factors that modify penetrance and severity of these conditions have the potential to provide novel answers to an issue that is the subject of intense debate among scientists investigating genetic cardiac conditions. Most of the current research is focusing on genetic factors and only a few studies have started investigating non-genetically acquired factors (such as diet, inflammation, and exposure to pollution). Identification of factors that modify penetrance and severity of inherited cardiac conditions is also likely to provide further mechanistic understanding of these conditions.

Medium long-term beneficiaries

Clinicians and patients could benefit directly because any positive findings could be readily translated into the clinic and have the potential of substantially improving the treatment and risk prediction of these patients. In addition, identification of factors that modify the penetrance and severity of inherited cardiac conditions could lead to the introduction of non-pharmacological interventions to prevent the onset of these conditions.

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

Our current mouse models of genetic cardiac conditions are likely to be of interest to other researchers, so we would be open to collaboration and the sharing of materials to maximise the impact of our work.

We will maximise access to our outputs by publishing in open access journals. Data sets, including those with negative outcomes, will be made available to other researchers via the institution's data repository and other public databases.
We will share good practice in terms of surgical techniques, disease model development and in vivo analyses. We already collaborate with numerous research groups, both at our own institution and elsewhere, so we envisage that the outputs of the current work will feed into further refinement of techniques and research partnerships.

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.

The project will use mice, predominantly with genetic modifications which are similar to genetic defects that in human induce genetic cardiac conditions. We can therefore study the impact of these genetic modifications in the whole animal, allowing us to model the genetic cardiac conditions that occurs in patients with cardiovascular disease.

The mouse is a highly relevant animal model for understanding disease processes in the cardiovascular system. In comparison to humans the mouse heart has the same gross and cellular structure; the pressure and volume characteristics closely resemble the human situation; and the vascular system is similar in terms of structure, function and its response to changes in blood pressure.

In order to generate mice for use in experiments, we will have to maintain breeding colonies of genetically modified mice and will use adult mice in subsequent experiments.

Overall, the mouse is an excellent model to characterize the mechanisms responsible for the onset of inherited cardiac conditions, and hence develop treatment strategies which are needed to make progress in the field.

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

Typically, mice will be bred to carry a genetic alteration followed by subsequent study of their cardiovascular function. These genetic alterations are similar to the genetic defect that cause genetic cardiac conditions and cause in the mice electrical and structural alterations which are very similar to the one detected in humans. We will study two main groups of genetic cardiac conditions:

Genetic arrhythmia syndromes that are characterized by abnormalities of the electrical properties of the heart that can cause irregular heart rhythms (arrhythmias)

Genetic condition of the cardiac muscle (cardiomyopathies) that are characterized by abnormalities in the structure and function of the heart that can cause heart failure.

A series of methods similar to those used to assess human heart and vessel (cardiovascular) function in the clinic eg blood pressure measurement, an electrocardiogram (ECG) and cardiac ultrasound will be used to analyse cardiovascular structure and function in mouse lines carrying genetic alterations. These analyses may be carried out at a single time point, or at multiple time points to analyse changes over time.

Some animals will have small wireless devices implanted to continuously monitor their heart rhythms and determine whether they develop irregular heart rhythms. These will be implanted under general anaesthesia into their abdomen.

In some animals, when it is not possible to deliver drugs mixed with food or water, we will implant minipumps to continuously deliver drugs for 2-3 weeks. These minipumps will be implanted under general anaesthesia in their abdomen or under the skin.
In some of these experiments, we will assess the impact of an intervention. In some experiments, the mice will receive a potential therapeutic drug by injection or mixed into their food or though minipumps to asses whether the drug prevents arrhythmias or prevents the structural and functional abnormalities produced by the cardiomyopathy. In other experiments, we may feed mice modified diets with high fat and sugars or with high content of fish oils to determine whether these diets modify the severity of arrhythmias or the structural abnormalities that the mutations cause. In other set of experiments mice will be infected with influenza virus or similar virus and will have injections to prolong the infection and the inflammation associated with it to determine whether prolonged infection and inflammation modify the severity of arrhythmias or the structural abnormalities that the mutations cause. In other experiments, we will expose the mice to pollutants to determine whether exposure to pollution modifies the severity of arrhythmias or the structural abnormalities that the mutations cause. Finally some mice will be fed a diet without potassium and will be given 'water' tablets to reduce the level of potassium in the blood and induce arrhythmias. These experiments will enable us to determine how low potassium causes arrhythmias and test novel treatments that in single cells have been shown to prevent arrhythmias induced by low potassium levels.
To perform genetic testing of the mice with genetic modification, we will have to extract DNA from animal tissues. The tissue will be obtained with one of the following methods: 1)

small ear clipping 2) blood sample, 3) hair sample 4) mouth swab.
At the end of each study described above, tissue will be collected after humane killing of mice for cellular, molecular, histological and in vitro analyses.

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

The genetic modifications that cause genetic arrhythmia syndromes do not affect the general wellbeing of the animals. They predispose the animals to have irregular heart rhythms when the animals are exposed to environmental stressors that cause physiological stress. These arrhythmias are well tolerated by the mice.

The genetic alterations that cause genetic cardiomyopathy can lead to the development of signs of heart failure. Genetic cardiomyopathy mice will represent 30-40 % of the mice utilised. The signs of heart failure include: respiratory difficulty, lethargy, cold tail, decreased interaction with other mice and lack of interest in food and water. Animals showing signs of heart failure will be promptly humanely killed.
Animals tend to tolerate arrhythmias well and the induction of arrhythmias should not be associated with significant adverse effects.

We expect the majority of animals to make a full recovery from surgical interventions conducted under anaesthesia. Post-operative pain will be prevented by administering analgesics. Some animals may not recover from anaesthesia as a result of surgical complications, and as such will feel no pain or distress.

High calorie diet (with high fat and sugars content) can result in obesity which can be associated with excessive grooming of fur and increased risk of infections.
Exposure to prolonged infection and inflammation causes signs of ill health that are typically characterized by weight loss (up to 15%) decreased food intake, decreased interaction with peers and other behavioural changes.

Similarly exposure to environmental pollutant such as phenanthrene and other derivatives of fossil fuels combustion can cause the same signs of ill health described above

Finally low potassium levels in the blood can be associated with dehydration and weight loss (up to 15%).

Expected severity categories and the 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 level associated with the breeding and maintenance of the genetically altered mice as expected to be mild for the genetic arrhythmia syndromes mice and moderate for the genetic cardiomyopathy mice.

Where animals undergo a surgical intervention with recovery from anaesthesia, we expect the majority to be within the moderate severity category.
We expect that modifications of diet should no cause significant adverse events and should be within the mild category.

We expects that chronic exposure to pollution, induction of sustained

infection/inflammation and induction of hypokalaemia will be within the moderate severity boundary.

In summary we expect 10-15% mild severity (genetic arrhythmia models who have diet modification or treatment with no surgery) 85-90% 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?

Non animal models of genetic arrhythmias syndromes and genetic cardiomyopathies (the two groups of conditions we want to study) provide some important and valuable mechanistic information however they have significant limitations that make the use of animal models essential.

In the case of genetic arrhythmia syndromes non animal model can provide information regarding what happens at the level of the single cells but offer very limited information on the interactions between cells that result in the generation of the irregular heart rhythms (arrhythmias) These can only be studied in the intact hearts. Similarly the assessment of novel treatments can only be performed in the intact heart.

Genetic cardiomyopathies are associated with complex structural and functional alterations of the heart. It is impossible to study and characterize these alterations in single cells. Therefore, the use of animal models and intact hearts is imperative in the study of genetic cardiomyopathies.

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

We have used isolated and cultured cells; heart muscle cells (cardiomyocytes) derived from stem cells generated from human tissues have been used to perform many of the experiments that underpin this project and we will continue to do so where appropriate. These studies mean that we are better informed to design our animal-based experiments.

Zebrafish and drosophila (fruit flies) are widely used in cardiovascular research. They are mainly used for identification of novel genes involved in cardiovascular function. However they provide limited mechanistic information and are not ideal for testing of novel treatments because the structure of the heart and the cardiovascular physiology are substantially different compared to humans.

Why were they not suitable?

Isolated cells are useful for providing proof of principle and for testing drugs (especially to determine tissue and circulating therapeutic levels) or understanding at the molecular level how signalling processes occur.

There has been a recent increase in the use of stem cells as an experimental model. There is a lack of human cardiovascular tissue available for research, which has led to the development of techniques to induce heart muscle cells (cardiomyocytes) from human stem cells. These cells have many of the characteristics of human cardiomyocytes and thus we are using them as a model system in which to characterise the effect of gene modification on hypertrophy and to understand the signalling pathways involved.

These in vitro systems can complement and enhance our research involving animals but will not act as a replacement for experiments, which require the understanding of gene function within the context of the whole organ and the whole body, especially in disease states.

Reduction

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

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

For each step of the project we have calculated the number of animals required to complete each experiment. This calculation is based on our own experience of similar experiments and/or from experiments reported in the scientific literature. We will use the minimum number of animals required to determine whether there is a difference between experimental groups.

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

In many cases we will use the animals as their own control.

Animals utilised for in vivo experiments will also be utilised for in vitro experiments. For in vitro experiments we will utilise each animal for more than one experiment.
We will utilise the PREPARE guidelines for planning and conducting high-quality research and testing on animals. We will utilise the NC3R's Experimental Design Assistant to design our experiments so that we are able to gain the most information from each individual animal.

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

The majority of animals used in this project will come from our own breeding colonies of genetically altered mice. In maintaining these colonies we will employ efficient breeding strategies to ensure that the number of excess animals is kept to a minimum. Colony size will be reviewed regularly to ensure that breeding matches the anticipated demand for experimental animals.

We constantly monitor whether to continue with a particular objective or not. Thus, if an experimental outcome indicates that there is no value in continuing, that aspect of the work

will cease.

Refinement

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

Which animal models and methods 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 two main mouse models of genetic cardiac conditions: Models of genetic arrhythmia syndromes and models of genetic cardiomyopathies.

Models of genetic arrhythmia syndromes: During our previous licence we have been able to establish that mice tolerates arrhythmias well. Arrhythmias do not tend to be associated with signs of distress.

The models of genetic cardiomyopathy develop significant cardiac structure and function alterations that can lead to sign and symptoms of heart failure. In some models it will be inevitable to observe some signs of heart failure because they are closely associated with the structural and functional alterations. We will perform regular assessment of cardiac structure and function in order to prevent or minimise the onset o heart failure.

All surgical techniques are carried out under general anaesthetic to ensure that the animal does not feel any pain, and any post-surgical pain is treated with the use of analgesics. In the post-surgical period animals will be closely monitored to detect signs that suggest pain is not well controlled. These signs include piloerection of fur, hunched posture, decreased activity and decreased interaction with peers. Any animal in which pain is uncontrolled, or which has significant surgical complications, or whose general health deteriorates, will be humanely killed. We have found optimal ages and weights for particular surgical procedures and we keep within these parameters.

Physiological analyses (eg ultrasound, imaging, ECG) are performed under general anaesthetic, in the majority of cases the mouse is under terminal anaesthetic from which it does not recover.

Any stress caused by administration of pharmacological agents is momentary as the injection is given. Where applicable we will aim to administer drugs mixed in diet or drinking water. If necessary mini- osmotic pumps will be used to administer pharmacological agents. Although their use initially involves minor surgery, the technique in our experience leads to highly reproducible and consistent results requiring fewer animals per experimental group.

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

While non-mammalian models are available, such as drosophila and zebrafish, there are key differences in their cardiovascular physiology compared to humans (they do not possess a four chambered heart and have a different circulatory system). Whilst we do not

use these model organisms directly in our own work, we keep abreast of current findings using these models through discussions with colleagues and through the published scientific literature. We need to utilise adult animals because the full phenotype of genetic arrhythmias syndromes and genetic cardiomyopathies is only present in the fully developed adult heart.

Some procedures are performed under terminal anaesthesia to remove any form of animal distress.

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

Over the course of the previous licence we have made a number of refinements to minimise harm to the animals used.

Refinements to anaesthesia and surgical approaches
We will adopt surgical techniques and processes that fully incorporate current best practice .

Monitoring
Detailed study plans are drawn up for each experiment and named persons consulted. This allows us to readily monitor and question the benefit of each mouse added to the study.
Regular appraisal of surgical outcomes. Continually refine the monitoring documentation to aid in assessing mouse welfare

Telemetry implants
We will utilise wireless telemetry implants so that there will be no need to tether the devices and there will be no wound exposure.

Minipumps implantation
We will aim to deliver most of the drugs mixed with food. When this is not possible, we will utilise minipumps that will be implanted either under the skin or in the abdomen. These will be implanted by fully trained staff utilising current best practice.

Arrhythmia induction studies
The type or arrhythmias we intend to study occur mainly when the heart is stimulated by adrenaline and caffeine. Over the last licence we observed that it was very difficult to cause arrhythmias with adrenaline in animals under general anaesthesia and these studies had to be in conscious animals. In addition we observed that arrhythmias in conscious animals are not associated with significant signs of distress and the animals recover fully from arrhythmias within 1-2 hours.

On the basis of these observations, we concluded that we can perform two arrhythmia challenges in each animal. This will allow us to perform one arrhythmia challenge while the animal is on treatment and another arrhythmia challenge while the animal is not on treatment. This will enable us to use each animal as its own control. This will produce more solid data and will reduce the number of animals needed to complete the study.

Animal housing
All cage enrichment will have adequate size entrance holes to avoid risk of skin catching/rubbing following implantation of the telemetry device/osmotic pumps and mice will be allowed to recover for at least 7 days after implantation of telemetry devices.

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

Procedures will be undertaken in accordance with institutional standard operating procedures (SOPs) and guidelines.

The approach to surgical procedures will be further informed by the Laboratory Animal Science Association's (LASA) Guiding Principles for Preparing for and Undertaking Aseptic Surgery (https://www.lasa.co.uk/wp-content/uploads/2018/05/Aseptic-Surgery.pdf).

The administration of substances will be performed according to the guidance provided by the joint working group for administration of substances. (Morton DB et al Refining procedures for the administration of substances Lab Anim. 2001 Jan;35(1):1-41.)

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

We seek to stay informed of 3Rs advances through innovations published in the literature, discussions with colleagues at our own and other institutions, regular updates provided by the establishment 3Rs Manager and through NC3Rs webpages newsletters.

27. Cow immunisation for milk derived antivenom productions

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

Venom, Envenoming, Cattle, Neglected Tropical Disease, Immunoglobulin

Animal types	Life stages
Cattle	pregnant, neonate

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim of this project is to examine if the immunisation of dairy cattle with snake venom can result in venom neutralising antibodies being detected in colostrum and milk.

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

Why is it important to undertake this work?

Antivenoms are serotherapies which are manufactured by refining antibodies from the antibody rich animal plasma of hyperimmunised animals, usually horses. However, this is a costly and invasive process which ultimately provides limited amount of material for serothearapry production. It is important to investigate the potential for other sustainable

sources of antibodies for serotherapies which will ideally also improve the welfare of animals in antivenom manufacture.

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

We hope to ascertain if serotherapies can or cannot be produced from the milk of hyper-immunised cattle.

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

There are two main long-term impacts if the project is ultimately successful (5-10 years).

Patients: The ability to generate serothearapy at lower cost and in substantially higher quantities will hopefully lead to an increase in availability and accessibility of these essential medicines globally.

Animals: Horses kept for serotherapy production are frequently bled for their antibody rich plasma. This invasive procedure can lead to anaemia and other complications if not properly performed. If successful, we may be able to replace the invasive collection of antibodies from plasma from horses with a non-invasive collection of antibodies from milk of cows.

To our knowledge, venom immunisations have not been performed on pregnant animals before, thus the fundamental research performed here has potential to inform future maternal-fetal research.

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

If successful, we will collaborate with industry to translate the findings towards product development and clinical study.

If unsuccessful, the work will be published in an appropriate journal to ensure work is not unnecessarily repeated in the future.

Species and numbers of animals expected to be used

- Cattle: 4

Predicted harms

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

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

Dairy cattle have been bred to produce large volumes of milk daily. The cows we use will be approximately 3 months pregnant when enrolled on study. As calf gestation is approx. 9 months, this will allow six months of venom hyperimmunisation before birth, which will allow for the cows to develop antibody responses which are efficient in neutralising venom activity. Once the cows have given birth, colostrum and milk samples will be examined to investigate the presence of anti-venom antibodies and to also investigate if any immunising venom toxins can be detected in milk.

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

Pregnant cows will be immunised on several occasions with very small, non-toxic quantities of venom. Small amounts of blood will be taken monthly to examine the antibody response to venoms. Once the cows calve, colostrum and milk samples will be taken during normal milking, while small amounts of blood will continue to be extracted monthly.

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

Venom and/or adjuvant may cause inflammation at the injection sites, similar to routine vaccination, but this should be transient. On occasion this can lead to the development of ulcers at the injection site, which should be short lasting and resolved with normal veterinary care. No systemic adverse effects (e.g. potentially lethal and painful effects of envenoming) are expected. Blood sampling will be performed as if it were routine veterinary care and should only have very mild and transient pain/inflammation. Anaphylaxis is a potential issue but very rare in antivenom production and we will carefully monitor after immunisation. Although it is possible that cross-reacting antibodies will be produced that cause a placentitis, or pyrexia that is induced may lead to abortion we do not anticipate 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)?

Expected severity for protocol 1 is moderate due to the potential of ulcers developing at injection sites. Expected severity for protocol 2 is mild.

Proportion of animals in each category is 50% moderate and 50% mild.

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

- Killed

Replacement

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

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

Poly-specific serotherapies can currently only be generated in animals through their natural response to immunisation.

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

Non-animal alternatives for antibody production are available, such as phage or yeast display.

Why were they not suitable?

Whilst we are actively pursuing these non-animal routes for antibody production, currently, these technologies are not at an advanced state of readiness to be able to be effective against multiple antigens simultaneously and are yet to enter any sort of clinical study for envenoming. This means animal derived serotherapies are likely to remain essential for treating envenoming for decades to come. We believe that, whilst not currently possible to replace the method of serotherapy production, it is possible to refine its production using the approach in this proposal.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 chosen to use 4 cattle for this body of work.

Two pregnant heifers will be used for immunisation. This is based on previous experience of experimental serotherapy production, the scale required for an early-stage proof of principle project and practical considerations at the establishment where the work is to be performed. Once these animals calve the calves will also be studied to observe and effects of exposure to immunisation whilst in utero or consumption of colostrum and milk containing anti-venom antibodies.

The other two cattle will consist of the calves born to the cows during the course of the project. Calves will have blood samples taken to monitor antibody and antigen transfer.

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

Two animals is the absolute minimal number we can use for this pilot work without introducing substantial risk (e.g. one animal not seroconverting to antigen).

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

This is a pilot study to show proof of principle and inform the number of animals which may be required for future production, which depends on antibody yield, in the event this project is successful.

Refinement

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

Which animal models and methods 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 methods described are relatively non-invasive compared to current methods used during normal veterinary care of the animals (e.g. immunisation, blood sampling). None of the methods are expected to cause suffering, distress or lasting harm to the animals. None of the methods are expected to cause more than transient mild pain/discomfort.

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

Dairy cattle are the most appropriate model as they are the animal to be used if the project is ultimately successful and translatable.

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

We will take blood samples at the minimum frequency expected to allow seroconversion to be monitored. Immunisation frequency is that expected to be successful over the time period.

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

We will follow relevant published government, world health organisation and veterinary and laboratory animal association guidelines for ensuring 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?

The PI is currently a recipient of an NC3Rs project grant and as such is obliged to not only remained informed about advances in the 3Rs and implement them, but to champion them to wider audiences as well.

We will stay informed through subscribing to both the NC3Rs and LASA websites, routinely attending NC3Rs seminars, and attending (and presenting) at suitable conferences (e.g. International Conference on Laboratory Animal Science and Welfare).

The person responsible for the project on the farm is a practicing Cattle veterinarian, European and UK Royal College of Veterinary Surgeons recognised specialist in bovine health and teaches aspects of cattle care to veterinary undergraduate students. As such, they are required to keep up to date with advances in cattle welfare and implement them effectively at all times.

28. Immuno-regulation during parasitic worm infection

Project duration

5 years 0 months

Project purpose

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

Key words

Helminths, Immunity, Immunoregulation, Co-infection

Animal types	Life stages
Mice	juvenile, adult, embryo, neonate, 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 research is to gain a detailed understanding of the relationship between parasitic worms (helminths), their hosts and their hosts' microbiome (all the bacteria/protozoa/fungi that naturally reside in the intestine). This includes understanding how the host responds to these helminth infections, how the immune response is able, or unable, to control these infections and the complexity and consequence of these responses in the light of other infections. Additionally we aim to understand the effects of treatments such as vaccination, changes in the host intestinal microbiome and changes in host diet on the ability of the host to mount an effective 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?

It is important to undertake this work as helminths are a neglected but medically and veterinary important group of pathogens that are ubiquitous in man and animals throughout the life-course and across the globe causing considerable ill health and disease. Control measures for these parasites are far from effective and there are no vaccines for use in humans and only a handful for domestic stock/companion animals. The impact of helminths on wider host physiology, associated diseases and pathologies and responses to other pathogens is under appreciated and neglected. Critically, animal models provide an opportunity to discover, define and test the mechanisms controlling immunity and disease that are impossible to perform *in vitro* or in humans and will pave the way for development of new treatments and control measures.

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

The research in this project will generate important new information on how the immune system controls and clears helminth infection or does not control helminth infection and regulates chronic infection. Also, it will have identified how this regulation is impacted by co-infection or changes in diet or in the microbiome. It will also have identified experimental vaccine candidates for some of the helminth infections we study. We will also have generated important new information on molecules that some helminths produce to manipulate the host immune system and we will have assessed their capacity to moderate other inflammatory conditions such as allergy. Direct outputs from the work will be peer-reviewed research articles, dataset resources that will be shared with the research community, and presentations at national and international meetings where we will disseminate our discoveries.

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

This will be of major benefit to researchers and scientists working on immunity to infection particularly helminths but also the broader field of immunity to pathogens and immunoregulation in general. In the short to mid-term, it will provide the rationale for developing new therapeutic approaches to controlling helminth infection and managing the consequences of chronic infection. Ultimately this research will be of benefit to the billions of humans currently infected with helminths worldwide.

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

We will publish our data in peer reviewed journals in open access format. We actively collaborate with research groups across the world that enhances the impact our findings and we will continue to grow new collaborations. We will publish negative data and unsuccessful approaches both as online supplemental data and in peer reviewed journals that accept robustly performed and analysed data regardless of impact.

Species and numbers of animals expected to be used.

- Mice: 12,500
- Rats: 125

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 host species and life stage to be used is the adult mouse. The mouse is the most well- defined immunological model system available that has remarkable similarity to the immune system of humans. Moreover, the general physiology of the mouse has multiple similarities to that of humans.

Importantly, rodents are naturally infected by the majority of the parasite species used and have counterparts in man. One parasite species used is a natural infection in rats and so these will be used to maintain the life cycle but all experimental infections will be carried out in mice. There are many advantages of working with the mouse, the most important being the ability to manipulate the mouse in order to precisely identify critical components involved in disease processes proving their importance. The availability of an extensive array of tools e.g. antibodies specific for different mouse cell populations to precisely identify them, synthetic mouse proteins to stimulate the immune system and mice with alterations in specific immune genes make the mouse an unrivalled system to study. For studies of the microbiota the availability of mice that have no bacteria or microbes, so called germ-free mice, or mice that have only well-defined bacterial populations provide unique and powerful approaches to investigate the importance of particular microbes in immune responses. There is now extensive literature to show that data generated from mouse studies is applicable to helminth studies in man. Ethically, studies in humans are largely restricted to analyses of peripheral blood cells and will not reflect the responses occurring at the most common site of helminth infection (e.g. the intestine). In addition, varied host genetics, unknown infection exposure history and nutritional variation all compound to make it difficult to achieve immunologically meaningful results from naturally acquired human infections in nature. In the laboratory, conditions can be precisely controlled, longitudinal studies designed to study immunity to single or multiple infections, informative local immune responses monitored, and comparisons made between animals that expel their parasite burden and those that do not.

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

The general experimental plan will involve the use of wild type or genetically modified animals that will be infected with helminth parasites. This may be one or more infection events depending on the question being investigated. It is noteworthy that naturally, animals and humans are repeatedly infected with helminth parasites (often more than one species) throughout their life course.

Experiments will utilise male and female mice where possible. We have already published data on the influence of sex on immune responses to helminth parasites.
Depending on parasite/pathogen, up to two different routes of infection may be used. No more than two parasites/pathogens will infect an animal at any one time. Depending on the parasite/pathogen, the majority of infections will last for between 10 and 35 days. Some, however, will be longer e.g. in the example of “trickle infections” whereby low numbers of parasites will be given weekly for up to ten weeks to mimic the natural infection process.

The experimental design will vary depending on the questions being asked. In addition to infection, animals may be administered compounds that modulate the immune response such as antibodies, cytokines that drive different types of immune responses or immunosuppressants such as cortisone. These will usually be administered by a series of repeated intraperitoneal injections during infection.
Animals may be immunised, usually subcutaneously, with antigens together with an adjuvant (a compound used to increase vaccine efficacy), usually on 2-3 occasions (i.e. vaccination), prior to infection. In some experiments germ-free or germ-free mice colonised

with defined commensal bacteria will be infected with one species of helminth. In some experiments, mice may be placed on a modified diet (e.g., high fat) for several weeks prior to helminth infection. Cumulative effects of multiple treatments will be minimised and although most treatments are minor, animals will be allowed to recover between them. Following infection at pre-optimised time points, animals will be culled and multiple tissues taken for analysis.

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

Experimental infection with helminths in the models we use is either associated with no discomfort or with mild/transient discomfort most often associated with the infection procedure. For some helminths animals experience discomfort (may be moderate and may experience a small weight loss) for longer
e.g. for 2-3 days or up to two weeks and in these cases, animals are closely monitored and given Hydragel/softened food during this period. In experiments where non-parasite infections (e.g. influenza) are used, infection is associated with a predictable and manageable transient weight loss. Mouse condition will be monitored, and mice weighed 1-2 times daily during peak infection (days 3 to days 6-10 post-infection). Outside of peak infection (before weight loss and once mice start to regain weight), mice will be checked and weighed every 1-2 days until day 14 when infection will have been cleared.

Expected severity categories and the 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 be routinely monitored during the course of experiments and the severity of disease and level of suffering will be graded according to well-defined scoring systems. Overall, 90% of the animals are expected to experience mild suffering, and up to 10% may experience transient moderate suffering.

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

- Killed
- Used in other projects

Replacement

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

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

The questions we wish to address focus on how the host responds (appropriately or inappropriately) to helminth infection. These parasites are large complex multicellular animals that develop, grow and change their characteristics during the course of infection. As such they are exquisitely adapted to their environment. As yet, there are no tissue culture systems that allow the development of all the life cycle changes that helminths go through and animals are absolutely required to provide the appropriate environment for helminth growth and survival. Also, unlike some parasites (such as malaria), it is not

possible to freeze life stages of helminths to maintain the life cycle and thus animals are critical for this. To address the majority of the questions we are asking we need an intact immune system i.e. only present in a complete animal, to precisely define the complex interactions between different cell populations that are activated in infected tissues and which move to lymph glands where they respond and then migrate back to the sites of infection such as the intestinal tract to carry out their protective and regulatory effects. Moreover, infection or immunization at one site of the body can influence host physiology at another site e.g. gut infection can influence brain inflammation. The intestinal microbiota of the host has a major influence on the host immune response. To date this complexity cannot be accurately modelled in tissue culture. There are no well-established models of human helminth infection in animals of lower sentience than mice that can be used to carry out this work.

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

As helminths can only be obtained from infected animals, we are constrained by this fact. We can, however, utilise specialised tissue cultures of host cells and parasites to answer very specific questions about the parasites and/or their interactions with isolated cell populations and/or immune molecules.

These approaches can sometimes be helpful in deciding a strategy to subsequently employ in animals. In collaboration with colleagues a completely new tissue culture system (organoids) has been developed for one of the helminths we study.

Encouragingly, for the first time, it permits infection by this species and some limited development of the parasite. The system does allow us to investigate specific interactions between parasite and host intestinal cells where they live and ask questions that we can subsequently take into animals in a more focused manner. It is hoped that as the organoid systems develop further, we can build up the complexity of the system (i.e. adding other cell populations such as immune cells and other supportive cells) which should be more informative.

Animals will still be required as a source of cells for the organoid cultures.

Why were they not suitable?

Although the *in vitro* systems allow certain questions to be addressed, this is only in a very specific and limited way. Moreover, the cells and organoid systems still rely on animals as a source of cells. The major limitation, however, is the complexity of the system that we are investigating. Host immune responses are the result of finely tuned multi-tissue responses that involve dynamic activities of cells and molecules from multiple tissues working in co-ordination to mediate effects. They are also influenced by different physiological systems such as the endocrine and nervous system and the microbiome. Moreover, helminths change location, in size, complexity and activity as they progress through development. Using mice, we are able to ask and answer very specific questions, addressing our objectives, within such a complex biological 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?

We have calculated the number of animals to be used based upon an estimation of the experiments that we propose to carry out, the number of repeat experiments required, the number of experimental groups involved in order to generate biologically significant results.This is based upon over twenty-five years' experience and data from the kinds of experiments proposed in addition to published data in the literature for similar experiments.

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

We utilise data from our previous published work to guide our required group size calculations. From extensive historical data we have a good idea of how many animals we will need to use to see significant changes between experimental groups in our experiments. We also use powerful statistical tests to help us determine animal groups sizes. For any new experiments where we do not have historical data we conduct pilot experiments to guide our decision making. We have identified optimum time points for taking tissue samples from the different models used which reduces unnecessary use of animals. We also follow ARRIVE and PREPARE guidelines for reporting of research involving animals, which outlines appropriate study design (e.g. control groups and sample sizes), how to avoid experimental bias, and the analytical framework for simple and complex experiments.

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

We select the most appropriate mouse strain to carry out our studies, based upon experience and the literature. We use both sexes where we can and record any differences in responses as metadata to examine going forward. We aim to use the most technologically advanced assays to collect the data ensuring optimum accuracy and sensitivity and assess as many relevant tissues as possible in a single animal including tissue archiving. Other tissues are offered to other researchers if they can be usefully used. We carefully manage our breeding colonies using the most efficient breeding strategy to generate the numbers of animals required. Any excess animals are offered to other researchers.

Interrupted breeding and cryopreservation of sperm/embryos are used as appropriate to reduce numbers. We take advice from the animal care staff who have expertise in maintaining colonies.

Refinement

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

Which animal models and methods will you use during this project? Explain why

these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice are the most appropriate model to use for our studies. Immunologically, they are the most well characterised defined species with a wealth of tools available to help precisely answer the questions we will be asking (e.g., genetically defined inbred and outbred strains, genetically engineered strains, intervention tools such as antibodies etc.). We also know that the different response phenotypes exhibited by different mouse strains reflects the variation in responses seen in outbred human populations. A small number of rats will be required to maintain one of the species of helminth used.

Our studies focus on helminth parasites, the majority of which are natural infections of wild mice. Thus, they are well adapted to their hosts and provide the most appropriate host species to study response in. In common with humans, helminth infections are naturally chronic in nature and most individuals harbour their parasites with no or minimal overt symptoms, the result of a dynamic immunoregulatory state. As a consequence, morbidity rather than mortality is the major consequence of infection, although some individuals following high levels of infection (e.g., children) or prolonged infections (adults) do suffer from pathological responses of infection.

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

Our research aims to inform on human health. The mouse is the least sentient species with the most appropriate physiology, including the immune system, which is remarkably similar to that of humans.

The parasitic diseases we research are caused by multicellular parasites that, in humans can cause considerable morbidity. We mostly use naturally occurring helminth infections in mice that reflect the major human infecting helminth species. There are no well-established animal models of helminth infection in lower vertebrates or invertebrates that accommodate either the relatedness of parasites or the host immune system.

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

In order to study the regulation of infection and the role of the immune system, it is necessary to infect animals with helminths/pathogens. Helminth parasites evolve to avoid being expelled from the host and control immunopathology that is subsequently generated. This is not perfect but, infections are mostly accompanied by relatively mild/moderate symptoms. Moreover, at certain stages of some helminth infections or in co-infections by non-helminths e.g., viruses or bacteria can cause transient discomfort. As will some of the treatments involving administration of substances or immunisations. We will continue to refine our assessment protocols for animal welfare taking advice from animal care staff and take action e.g., administration of local anaesthesia or analgesia (e.g., emulon before sampling blood from the tail vein) if indicated and provide modified diet e.g. Hydragel or softened food to encourage feeding for the short duration of symptoms or weight loss.

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

We will follow NC3Rs, ARRIVE and PREPARE guidance and we will continually assess our experimental design in relation to advances within the immunology and parasitology

literature.

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

We strive to identify and implement new experimental advances related to 3Rs in our work through current literature and in-house information via email, regular animal facility 3Rs driven information and training meetings and AWERB awaydays. We use an extensive set of in-house Standard Operating Procedures for animal work that are modified considering new developments related to animal welfare. We get personal formative and formal feedback on the 3Rs from project licence mid-term and retrospective reviews.

29. Improving the nutritional qualities and nationwide health benefits of milk via a novel, epigenomics-driven platform, EPIHERD

Project duration

3 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes.

Key words

Dairy Cattle, Epigenomics, Biomarkers, Machine Learning, Milk quality

Animal types	Life stages
Cattle	adult, pregnant

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

Aim of the project is identify biomarkers that effect nutritional quality of milk and other performance outcomes (i.e., milk yield) in dairy cows using epigenomics and machine learning.

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

Why is it important to undertake this work?

Consumed by 96% of UK adults, cow's milk is one of the most nutritionally complete foods available and contains 'nutrients of concern' lacking in many diets. For example, the United Kingdom (UK) has one of the world's lowest omega-3 intakes and continuous national survey of the UK’s dietary and nutritional intake (The National Diet and Nutrition Survey, NDNS) highlighted increasing omega-3 consumption as crucially important. Cow’s milk is a

significant source of key fatty acids, including conjugated linoleic acid (CLA) and omega-3s and -6s which have been linked to multiple health benefits, including reduced risk of diabetes, heart disease and depression. However, the nutritional content of cow's milk can vary considerably, as well as other outcomes such as production (i.e. milk yield) of dairy cows. Previously scientific research has focused on the genetic make-up, or presence or absence of specific genes, and how these can impact the nutritional quality of milk and production factors such as the amount of milk produced by a dairy cow. So far, this has only been able to account for 30% of the variation between animals and shows that there are still other factors causing differences between cows even with the same DNA. It is now known that the way these genes are expressed and behave (epigenetics) is the largest factor influencing the difference in outcomes (such as milk quality, and herd health and production) between genetically identical individuals. This presents a significant opportunity for epigenomics to help farmers improve milk quality and dairy cow productivity in response to increasing demand. Consequently, we aim to investigate how genes are expressed in a group of dairy cows across one full lactation period by collecting milk composition, cow health and production data. We will develop sophisticated data analytics (machine-learning/AI) to assess gene expression and identify biological markers. This will help us to understand how in the future these biomarkers may be able to accurately assess factors such as milk composition and milk yield. This is expected to be valuable for dairy farmers and enable them to make more informed decisions to improve milk quality (i.e., omega-3, fatty acid profile, protein, etc.), herd productivity (i.e. milk yield), and sustainability (e.g. reducing cattle GHG emissions via improved productivity) and breeding cows suited to their system. Additionally, the machine learning and data analytics methods developed will be applicable to many other areas of gene expression in both livestock and humans and can be further utilised in future work.

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

This project will used sophisticated data analytics (machine-learning/AI) to assess which genes are expressed in dairy cows over one full lactation period and if these may be linked to milk quality, and milk production. This will allow us to identify key biomarkers and validate their ability to be used as an accurate biomarker to assess factors like milk nutritional content, quality, and yield. Combining and linking the epigenetic data with the animal health and production data will enable dairy farmers to make more informed decisions regarding areas like improving milk nutritional content, or increased productivity and sustainability. The information and data obtained in this project will be highly beneficial to dairy farmers and will be collated into an innovative RNA-AI-platform enabling farmers to identify specific factors that influence phenotypic (observed) response in cattle (i.e., milk yield, longevity).

A further benefit is that the milk and blood samples collected throughout the project will create a biobank which would be available for use in future research. Similarly, the machine learning analysis used will be applicable to many other areas of gene expression in both livestock and humans and will be further utilised in future work. It is expected that the result of this project, positive or negative, will be published as peer reviewed papers in well-known scientific journals. Data generated under this project will be presented at multiple national and international conferences by the principal investigator, post- doctoral researchers, and PhD students.

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

This project, by identifying key biomarkers, will obtain knowledge and provide information that can be built upon to investigate and understand how epigenomic biomarkers relate to

the diversity in observable cow and dairy milk characteristics, and how they can be harnessed for improved sustainability, productivity and health for dairy cows. The information and outputs provided by this project will benefit dairy farmers, dairy cows themselves, and aid in improving human health via improved nutrition. One of the primary outputs will be the epigenomic platform which will enable dairy farmers to better influence key factors like herd performance, milk quality, and animal health. Long term benefits include helping farmers sell consistently higher quality milk to food manufacturers and, meeting consumer demands for healthier foodstuffs. Helping farmers identify best-performing animals will lead to increased profitability and thus sustainability of dairy businesses. It is expected that the outputs from this project will also benefit dairy cow health and welfare as the epigenetic insights provided are likely to help reduce culling due to poor production, it will allow strategic breeding so that cows are bred appropriate to the system and ultimately improved profitability of farm will allow investment into farm infrastructure and environmental enrichment improving the lives of cows.

The project is focused on dairy cows used to produce milk for human consumption, and therefore another benefit of this project will be the knowledge of how different biomarkers and epigenetic factors impact the nutritional quality and content of the milk produced. By increasing our understanding in this area, the outputs of this project have the potential to impact the nutritional content of milk, ensuring one of the most affordable, nutritionally complete dietary staples remains that way, and accessible-to- all (food security). For example, increasing the level of or consistency of omega-3 levels, a key fatty acid previously demonstrated to reduce the risk of diabetes, heart disease and depression.

Establishment of a biobank will provide a valuable resource with benefits to the research community for grant applications and research as well as the animals, farmers, advisors and consumers benefiting from outputs of this research. The biobank will be managed by the principal investigator (PI) and research group with storage of samples at -80oC and comprehensive cataloguing of samples. Access to samples and accompanying data will be available to the research group as well as internal and external research groups where appropriate for collaboration.

Furthermore, the scientific papers which will be published containing the results of the project will also address gaps within current scientific knowledge, as although there have been advances in the use of epigenetics in humans, there has been limited exploration in livestock species.

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

The project findings, both positive and negative, will be published in high-impact open access journals and presented at national and international conferences, seminars, and meetings. The findings will also be used to inform future research projects within the same area. Additionally, we will use established networks (e.g., AHDB Dairy (Agriculture and Horticulture Development Board), BCVA (British Cattle Veterinary Association), and other working groups) to disseminate research findings.
Where possible we will collaborate and share resources (samples, knowledge, technical skills) with other researchers within and outside of the University, to maximise the scope and impact of our research. It is our anticipation that data generated under this project (both positive and negative) will be made available.

Species and numbers of animals expected to be used.

- Cattle: 60

Predicted harms

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

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

This project follows a group of adult dairy cows from one farm over the course of one lactation period and aims to identify biomarkers that effect nutritional quality of milk and production outcomes (e.g., milk yield) using epigenomics and machine learning analysis. The project is specifically focused on cow's milk, which represents 81% of total global milk production (714 million tonnes), and therefore dairy cows are the most appropriate animal for this project. Cow's milk is specific to female dairy cattle and therefore the use of female cattle during a lactation cycle is necessary and no alternative is appropriate for this research.

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

The project will recruit and follow a population of 60 lactating adult dairy cows managed for milk production on one farm over a period of 12 months. Once cows have successfully transitioned into the milking herd they will be housed in appropriate groups to limit the disruption due to collection for sampling. Cows will be recruited around 2 months prior to expected calving date (heifers or mature cows) and deemed in good health using treatment records and visual inspection.

The cows will then be followed through the subsequent lactation for a period of approximately 10 months. Blood samples will be collected monthly under restraint (maximum of 12 sampling timepoints; equivalent to 12 months on trial). Two sampling points will occur prior to calving, then a maximum of 10 afterwards (totalling 12). Collection of samples will involve blood sampling from a superficial vein (restraint using appropriate handling facilities including head yoke, cattle crush, AI stalls). Milk samples will be collected monthly for a maximum of 10 months post-calving, totalling a maximum of 10 milk samples per individual. The milk samples will be collected from the milking robot which is used to milk the cows during their normal routine and therefore will not require any intervention or change in routine. Otherwise, cows will be housed and managed (including feeding, milking, health management) according to the normal farm management routines for the rest of the herd in high quality and spacious facilities. The number of sampling days will also be limited as appropriate to ensure all samples required are collected but batched to further reduce disruption.

Gene expression data from the blood samples will be related to cow performance indicators and milk quality, using machine learning and data mining. A library of temporal samples (milk and blood) will be established alongside meta data on health, behaviour and performance of the dairy cows. Records on behaviour, health, treatment and production parameters will be collated from the herd management software.

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

It is unlikely that any adverse effects will occur due to the procedures described. To ensure this we expect the majority of the samples to be collected whilst cows are housed in their

normal environment using normal husbandry methods of restraint (e.g. locking head yokes or stalls). Where it is more appropriate (e.g. collection of blood samples from an alternative site such as the jugular instead of the tail vein) or for a safety reasons, cows may be restrained in a cattle crush. Collection of blood samples from a superficial vein (tail or jugular) for epigenomic analysis may result in inflammation and/or soreness at the site of sampling. If this were to occur, it will be localised to the needle insertion site and the duration should typically be no more than 24 hours. Throughout the study period, all animals will be monitored for the duration of the study for general condition, signs of ill-health or general signs of distress.

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

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

As blood sample will be collected from all animals in this study, it is anticipated that 100% of animals will experience a procedure of a mild severity.

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

- Kept alive
- Used in other projects

Replacement

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

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

This research is specific to dairy cattle and therefore it is necessary to conduct this project with animals to achieve the aim of the project. 96% of UK adults consume cow's milk however, the nutritional content of cow's milk can vary considerably. Peer-reviewed research shows that classical genetics (presence/absence of a gene) only accounts for ~30% of complex traits outcome, leaving considerable phenotypic variability among genetically similar animals that cannot be explained by DNA alone.
Epigenomics (the expression/activity of a gene) is a field that can help complete the picture, being the biggest factor influencing the regulation of desirable characteristics in dairy cattle. This project aims to investigate how gene expression of biomarkers relate to nutritional quality of milk as well cow performance throughout a lactation cycle. This research is specific to dairy cattle and therefore it is necessary to conduct this project with animals to achieve the aim of the project.

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

No alternatives are possible for this project.

Why were they not suitable?

The project is specific to dairy cattle and requires the collection of dairy cow's milk and blood samples, and meta, phenotypic and performance data to assess biomarkers and

gene expression to achieve the project aim.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 conducted machine learning on gene expression pilot data from a small cross sectional pilot data obtained from project collaborator. The data has been used to calculate a required sample size for this project. Thus, the number of animals required for this project has been estimated based on RNA gene expression data which demonstrated that minimum sample size of 56 is required, thus sampling 60 as proposed in this study to ensure any drop-outs. This power analysis was done for studying gene expression with machine learning methods that will achieve the false discovery rate of only 5% to identify differential gene expression with regards to milk quality and yield. Sampling every month (maximum of 12 months for blood and 10 for milk samples) will allow us to test the differential gene expression over the course of one lactation period and how stable the expression is over lactation.

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

RNA gene expression data obtained from a cross sectional pilot study involving dairy cows has been used to calculate a required sample size for this project. Robust analysis of this pilot data enabled the accurate estimation of the minimum number of animals needed to maximise the likelihood of obtaining highly predictive algorithms for differential gene expression; this number is used in the current study design. Monthly repeat samples are to ensure that stability of gene expression can be tested over the course of the lactation.

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

A pilot data has been used to inform the design of this project. This project will result in the establishment of a biobank of samples from a cohort of lactating dairy cows that will provide a valuable resource for future research studies and aid in the generation of preliminary data for subsequent projects and grant applications.

Refinement

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

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

to the animals.

Dairy cattle are the most appropriate animal for studying the epigenomic links between cow's milk quality and performance outcomes. Holstein- Friesian cows have been chosen as this is the primary breed of cow used for milk production in the UK so the results of this study will be applicable to most animals in the UK. Throughout the work, animals will be group-housed to provide near-commercial environments and conditions to ensure findings are relevant to the UK dairy industry. The number of animals and sampling times has been decided upon as such that enough data can be collected to meet the project aim while keeping invasive procedures to a minimum. Our choice of animals and approach is the most refined for the intended purpose and the only possible method to meet our research aims.

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

The research is specific to dairy cows, milk production, and cow's milk nutritional quality. Therefore, it is necessary to conduct this research using dairy cattle to meet the project aim. It would not be possible to conduct the same research in another species, life stage, or model.

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

All cows will remain within the herd and their normal environment during the study period. Throughout the study, all animals will be monitored for the duration of the study for general condition, signs of ill- health or general signs of distress daily by farm staff and weekly monitoring by research staff. This level of monitoring will ensure any changes to condition, demeanour or behaviour or significant deviations from normal will be recorded, and where appropriate, result in discussion with the staff within the facility, the NVS or their deputy and appropriate action taken. The cows will be sampled within their normal home environment to minimise any welfare costs due to movement or separation from the herd. Furthermore, individual health and production parameters are measured at a high frequency via the robotic milking system which alerts farm staff to health and production deviations from normal. Any alerts will be result in appropriate discussions between farm staff and researchers.

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

Throughout the course of this project, we will make reference to multiple sources for best practice guidance including:
NC3R's website for details on experimental design: https://nc3rs.org.uk/experimental-design
NC3R's ARRIVE guidelines: https://www.nc3rs.org.uk/arrive-guidelines
-The UFAW Handbook on the Care and Management of Laboratory and Other Research Animals, Eighth Edition

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

There will be regular liaison with the local named animal care and welfare officer (NACWO) and named veterinary surgeon (NVS). Subscription to the NC3Rs monthly email alerts provide the most recent developments in NC3Rs publications, guidance and information.

We also receive regular updates and latest animal welfare guidance information via bulletins disseminated from our Biomedical Research unit.

30. The role of peripheral nervous system in oral mucosa development, homeostasis, and cancer

Project duration

5 years 0 months

Project purpose

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

Key words

Developmental Biology, Cancer, Peripheral Nervous System, Stem cells, Therapy

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

To understand the mechanistic role of the peripheral nervous system in oral mucosa development, homeostasis and tumourigenesis.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 oral mucosa is involved in the 6th most prevalent inflammatory human disease, periodontitis. Additionally, it is also one of the primary sites of head and neck cancer. There are currently 476,125 new cases of oral cancer diagnosed every year, and incidence rates have and are expected to increase in the next years. Despite its importance, the molecular and cellular mechanisms involved in oral mucosa homeostasis remain largely unknown.

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

Understanding the complex developmental programmes shaping the establishment of oral neural circuits and the intricate interactions that constitute their contribution to tissue homeostasis is an essential step for elucidating the pathogenesis of oral disease. Cellular, molecular, and mechanistic knowledge emerging from this study will provide new insights for the development of successful therapeutic and tissue regeneration therapies. This project strategy of pursuing discovery across multiple disciples, neuroscience, immunology, biology, machine learning and oral medicine will generate high impact research in the form of publications and academic meetings. Importantly, this project has the potential of opening a new research field in the study of neuroimmune/neuroepithelial interactions across developmental time.

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

On a short-term, this project will benefit the scientific community. I will be applying the latest genomics technologies to understand human disease, which will produce high quality data. These data will be publicly available, allowing other research and clinical groups to investigate and produce new hypotheses. This will accelerate the field and contribute to impactful and collaborative work.
On a long-term, new mechanistic knowledge emerging from this study will benefit clinicians and patients by determining novel mechanisms involved in disease pathogenesis. This research project will potentially lead to the generation of new drug targets for clinical use, for disease resolution, but also tissue repair. Current treatments for oral inflammatory diseases are limited to alleviating symptoms, rather than tackling the cause. Similarly, most patients with oral cancer are diagnosed in late stages without a clinically-evident antecedent pre-malignant lesion. Thus, a detailed molecular characterization and functional knowledge arising from our human and animal work will allow incorporation of prognostic and predictive biomarkers into clinical management to overcome obstacles to targeted therapies and enable prolonged survival.

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

To maximise the outputs of this work, we will create user-friendly public data portals for all bioinformatics analyses performed. This will enable both clinicians and scientists to easily access and analyse these data. By supporting an open and interactive research culture, with an outward-looking attitude, we aim to accelerate knowledge, ideas, and resources.

Species and numbers of animals expected to be used.

- Mice: Breeding - 1000; Experimental - 500.

Predicted harms

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

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

Mice have a peripheral nervous system that is sufficiently analogous to humans to enable and draw inferences between data derived from human data and mice genomics and

functional data.

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

Generating and maintaining genetically altered (GA) mouse models to study the role of the peripheral nervous system in oral mucosa physiology.

GA mice will have a specific mutation that do not lead to development of pathologies. Mutation(s) will determine the presence or biological activity of a cell, or regulate intercellular signals, or modify expression of a molecule.

Tissue biopsy to determine genetic status (e.g., by ear punch) will be performed. Administration of a labelling/tracing agent by generic routes such as subcutaneous or intraperitoneal.

Use of carcinogens to induce early stage of oral cancer development.
Oral dosing via drinking water of a chemical agent that may induce oncogenic changes in the oral mucosa. Then mice will be given normal drinking water until the end point up to 6 months from the start of the treatment. Brief anaesthesia (AB) may be required for tumour assessment in the oral cavity. Mice may be injected with innocuous cell trackers via subcutaneous intraperitoneally before killing to assess cell proliferation.

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

GA animals are not expected to develop a harmful phenotype; however, adverse effects may include transient weight loss (typically up to 15%), as reported by the dosing of some transgene activators, which recovers over time. Animals may show signs of general malaise, such as hunched postured, piloerection, inappetence, reduced mobility, unsteady gait and reduced general activity. Typically, this is transient and animals improve over time.

A small number of mice may develop a harmful phenotype due to ablation of a specific cell or signalling molecule. Therefore, all mice will be monitored closely and any mice where a harmful phenotype becomes evident and likely to exceed moderate, will be terminated.

GA animals treated with an oral carcinogen will develop oral, oesophageal and potentially forestomach tumours. Our research interest is targeting the onset and early development of cancer; yet, due to the internal nature of these early tumour developments, most of these mice will show gradual 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)?

GA animals are all expected to develop a mild phenotype. As mentioned above, our studies are focused on the early stages of tumour development, thus most GA animals treated with an oral carcinogen may develop a moderate phenotype. Specific monitoring, and care protocols to regularly assess body weight and clinical welfare parameters will be carried out (weekly to daily as tumour onset and development is timely expected). Humane endpoints (e.g., body weight loss up to 15%) will be applied to ensure no clinical signs will progress beyond 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 core of our research is done on human samples, however, we require animal models to functionally test new generated hypotheses and gain mechanistic knowledge. To use the genetically modified animals provides a unique approach for such mechanistic studies; furthermore, some of the interactive modelling approaches between the autonomous nervous system and the oral mucosa remains complex and not been able to fully replicate by *in vitro* cellular systems. Importantly, our animal studies are supported and well targeted by initial human clinical and tissue/cellular/genetic bench data.

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

We consider complex *in vitro* 3D systems, such as self-sustaining 3D epithelia cultures (Jones *et al*. 2023) and oral mucosa organoids (Clevers *et al*. 2019). These allow early bench and screening studies, yet cannot recapitulate full *in vivo* complex communications between the peripheral nervous system, immune system and the specialised oral mucosa. We will also use computer modelling to test some hypotheses *in silico.*

Why were they not suitable?

Despite being complex study systems and good models for tumour-microenvironment interactions, they do not allow the study of neuronal interactions in early tumourigenesis and inflammation; therefore, there is currently a continued requirement for mouse models. We aim to work in collaboration with other groups to introduce more complexity into these established systems. *In silico* models offer generation of robust candidate predictions for some of our working hypotheses; however, require further *in vivo* validation.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 experiments have been designed with reference to the ARRIVE guidelines published by the NC3Rs. We will ensure that all animals are maintained on an applicable genetic background and housed under the same conditions with environmental enrichment provided. By using matching mouse strains, we will be minimizing variation between experimental groups and maximise reproducibility. Where applicable we will constrain groups to age, weight, and sex matched animals. This does not restrict groups to single

sex animals but makes sure that equal composition of groups is maintained.

During the experimental design we will engage with a statistician to ensure that we have the clear strategy to generate and process the resulting data. At every stage we will challenge our data sets to screening raw data for obvious errors such as imputing/typographic errors and analysis of variance with comparable, existing datasets. All raw data will be recorded and archived appropriately to allow follow-up statistical studies to minimize animal usage.

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

To support our experimental design strategy, improve scientific efficiency and minimise animal use, we used the strategic planning for research programmes developed by FRAME, and the NC3R's Experimental Design Assistant. We further used the 3Rs databases ALTWEB and AWIC to search for alternatives to Animal Testing and improve animal care strategies.

Importantly, all animal studies will be informed by our human oral mucosa datasets (including developmental studies); therefore, we will substantially reduce the need for animal data in our Discovery research. Additionally, by using human data for exploratory research, we will have high quality data to test only meaningful and essential mechanistic hypotheses relevant to human health.

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

Breeding colony management guidelines from NC3Rs and the Jackson Laboratory will be followed to optimise our breeding strategy, with appropriate controls to ensure the strain genetic integrity and all the appropriate records.
Pilot animal data will be shared from other research groups (e.g., tissues) to allow optimization of specific experiments (e.g., antibody testing) without the need to sacrifice animals. Additionally, suitable publicly available data will be used and analysed to inform any of our studies (e.g., sequence 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.

We have elected to use the mouse as our *in vivo* model as it is exceptionally well characterized in terms of its genome, physiology and pathology. Mice and humans share virtually the same set of genes, with the mouse genome being as well understood and annotated as that of humans.

Importantly, mice have a peripheral nervous system that is sufficiently analogous to humans to enable us to draw inferences between data derived from human data and mouse mechanistic data. Mice are the most appropriate choice of species for this research due to their oral anatomy and peripheral neural networks, which are similar and largely comparable to the human oral tissues. Similarly, our understanding of the processes regulating nervous and immune systems is more advanced in this species that in possibly any other vertebrate. As such, we will be able to integrate mouse transcriptomic, imaging, and physiological data from this animal to a higher degree than would be possible in other systems, whilst having full control over environmental and experimental variables.

Moreover, murine models also afford the opportunity of targeted gene modification to facilitate mechanistic studies in a mammalian system. Together, these factors will ensure that outputs from our proposed research are translational to humans.
GA lines are not expected to develop any harmful phenotype and are all well established between two project collaborators. Transgene activation may induce temporary discomfort and body weight loss; however, our team is well familiarized with the care and monitoring of these animals. We will aim to use refined approaches for transgene activator dosing (e.g., oral gavage, minimize carrier volume) of adult mice. The protocols of GA-inducing drug and sometimes dosing non-hazardous labelling cells substances are highly unlikely to cause adverse events of more than mild to temporary moderate severity. These protocols are the most efficient to achieve the aims based on our full literature-search. If a more efficient and less invasive protocol is established somewhere, it will be used. Unless there is a critical scientific justification, substances will always be administered by the least invasive route for the shortest duration possible.

The protocol we will use to induce oral cancer is well established and characterised; previous genomic studies have demonstrated that the mutational signatures and tumour architecture of human and mouse overlap extensively using this method.

Thus, data and mechanistic studies originating from this model will provide a highly reproducible foundation to study human disease. Importantly, we are only interested in the onset and early development of oral cancer, therefore severe clinical signs are not expected. However, our team has extensive expertise in the expected clinical signs and cancer staging progression, such as body weight loss, piloerection, and hunching posture onset. To ameliorate animal distress, familiarisation of the animals to handling during check and the use of short exposure to anaesthesia (preferably via inhalational agents to provide quick recovery). Measures supporting maintenance of body temperature, provision of oxygen and protecting cornea from damage by use of a protective eye ointment will contribute to overall refinement of anaesthesia. We will always provide high standards of post-operative care by developing a protocol sheet with all details of the procedure and aftercare needed. When necessary, analgesia will be provided to minimise pain and distress.

We have extensive prior experience of most substances we expect to administer as part of these protocols including optimal dosing strategies and information on tolerability and efficacy. Whenever any new substances are to be used, small scale pilot experiments adapted from existing data will be performed to confirm efficacy (using assays and humane endpoints appropriate to the substance) and identify any adverse effects to minimise any mild to temporarily moderate severity.
Substances will always be administered in a sterile form. Where possible clinical grade compounds will be used.

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

It is essential that any genetically altered animal model used in this programme of study to reflect as closely as possible the human disease or allow us to examine the functions of genes within that disease. Thus, our choice of GA models will always follow this objective to answer relevant questions for gaining a deeper understanding of human cancer.

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

For each breeding and experimental procedures, we will develop specific protocol monitoring, pain management and recovery sheets in accordance with best current guidelines (NC3Rs, LASA).

Gentle handling and training reduce stress-related behaviours will be implemented during research. We will design a welfare monitoring tool for our studies in discussion with the named persons to identify any early clinical sign/signal and its progression. We will always provide high standards of post- operative care by developing a protocol sheet with all details of the procedure and aftercare needed.
Housing and husbandry conditions, including the physical and social environment in which mice are housed will be established in accordance to NC3R's and LASA guidelines. By following these standards, we will optimise both the welfare and the quality of research data. Group-housing and enriched environment such as tunnels, boxes and/or climbing structures will be provided to promote social exploration and naturalistic behaviour. Suitable nesting material (e.g., shredded paper strips, nestlets, wool woll) will be provided to ensure good nest for pup wellbeing.
To apply appropriate humane endpoints (and effective cost-benefit analyses of projects) we will establish and use objective assessments of clinical signs and tumour progression and the affective component of pain in collaboration with experienced project licence holders and/or based on current literature.

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

N3CRs, including experimental designs (https://www.nc3rs.org.uk/our-portfolio/experimental-design- assistant-eda) and colony management (https://nc3rs.org.uk/3rs-resources/breeding-and-colony- management/colony-management-best-practice), PREPARE (https://norecopa.no/prepare), FRAME (https://frame.org.uk/resources/experimental-design/) and ARRIVE 2.0 for transparency in reporting (https://arriveguidelines.org/arrive-guidelines) guidelines will be used.

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

To actively follow and implement the latest 3Rs advances, we will keep up to date via the NC3Rs resource library updates, UAR (Understanding Animal Research), LASA, NORECOPA, FRAME (Fund for the Replacement of Animals in Medical Experiments). We will seek other 3Rs related databases, including ALtweb (www.altweb.jhsph.edu) and CAAT (Center for Alternatives to Animal Testing; http://caat.jhsph.edu/about-caat/). We will continuously seek help by following UFAW, LASA Best Practice Guidelines series, and ECVAM (European Centre for Validation of Alternative Methods). A review of these datasets will be added to our lab meetings' agenda. We will also always engage with other project licence holders to identify novel methods and training needs.

31. Mechanisms of change in HPA activity

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

Neuroendocrinology, Stress, Hormones, Glucocorticoids, Biological rhythms

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The overall aim of this project is to generate mechanistic understanding of how and why the activity of the HPA axis changes with physiological condition.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 hypothalamic-pituitary-adrenal (HPA) axis is a vital system in the body controlling the release of "stress hormones" such as cortisol. Oscillations in this hormone are critical for our health as they control the activity of many important biological functions and ensure the body is in an ideal state to respond to stress. These hormone oscillations change according to physiological state (e.g. gender or ageing). Moreover, stressful experiences early in life, or excessively-large or prolonged periods of stress, can lead to long-lasting

disruptions in HPA axis activity, which in turn has consequences for brain function and physical and mental well-being. Characterising the mechanisms that regulate the dynamic activity of the HPA axis is a critical step towards understanding how and why the dynamic activity of this system becomes dysregulated.

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

The expected outputs at the end of this project are: (i) data providing novel insight into the mechanisms through which the HPA axis regulates hormone secretion; (ii) mathematical models describing the activity of the HPA axis; and (iii) associated publications of these.

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

In the short-term, the outputs generated by this programme of work will be used by our research group to steer our future research directions, as well as our scientific collaborators who work on different, but related, aspects of HPA axis function. Through presentations at national and international scientific conferences and publications in academic journals, the wider community in the fields of stress, mathematical biology, and basic/clinical neuroendocrinology will have the opportunity to use our outputs to guide their own research programmes. In particular, many other hormonal systems display rhythms of activity with functional consequences, including systems controlling the secretion of insulin, growth hormone, and sex steroids; the experimental and mathematical modelling approaches we will employ in this project, as well as the data generated using these approaches, will benefit researchers working on these other related neuroendocrine systems. Through public outreach events, such as public talks (scientific talks in a lay format) and patient feedback workshops, the public and patients undergoing hormone replacement therapy or treatment for stress-related conditions will have the opportunity to learn about and inform some of the basic research being carried out in this field.

Beyond the term of this project, the potential benefits are that the data generated may have far- reaching implications for the treatment of diseases associated with disrupted HPA dynamics. This not only includes disorders of the HPA axis such as Addison’s disease, congenital adrenal hyperplasia, and pituitary deficiency, but also stress-related disorders. Characterising the mechanisms that regulate the dynamic activity of the HPA axis is the critical first step towards understanding how and why the activity of this system becomes disrupted in these conditions.

Our findings may also have the longer-term potential to benefit patients requiring hormone replacement or steroid therapy for inflammatory or malignant disease. Despite what is now known about the importance of glucocorticoid oscillations, many patients are typically still being exposed to constant levels of potent, long-acting, synthetic steroids, which may well limit efficacy of treatment as well as contribute to the high levels of side effects associated with long-term use of synthetic steroids. The data generated by this programme of work will accelerate the development of novel chronologically-based methods of drug administration that mimic more naturally the body's own dynamic production of glucocorticoids; this in turn should improve efficacy and decrease side effects from glucocorticoid administration. Since steroids are one of the most widely-prescribed classes of drug in the UK, reducing their side effects will not only benefit patients, but will also result in significant financial savings.

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

We will ensure our project findings reach the global scientific community in two key ways:

Through the publication of our data and research findings (successful and unsuccessful) in open- access, peer-reviewed biomedical journals; as part of this process, our datasets and mathematical modelling and analysis code will be made publicly accessible.

Through the presentation of our data at local seminars, as well as national and international scientific conferences attended by both basic and clinical scientists; this will typically take the form of oral and poster presentations.

We will continue to foster our national and international networks of scientific collaborators, sharing the findings (e.g. data; knowledge and insight; methodological updates) from this project in a timely manner.

Species and numbers of animals expected to be used.

- 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.

The rat is the most widely used animal for studies into the function and control of the HPA axis, and its HPA axis has been more thoroughly characterised than that of any other species. This is due, in part, to the fact that the HPA axis in rats and humans shares many similarities, including a circadian and ultradian dynamic pattern of hormone secretion. The rat is therefore a valuable model in which to answer questions that cannot, for ethical reasons, be addressed directly in humans. The experimental methods used in this field of research are well tolerated by the rat, and do not typically result in overt signs of distress.

The mouse is not suitable to use in this project. Compared to the rat, far less is understood about the function of the mouse HPA axis in both unstressed and stressed conditions; one of the main reasons for this is that the low total blood volume in the mouse precludes high-frequency blood sampling for quantification of HPA-related hormone secretion.

In some studies, we will specifically use male rats since their endogenous HPA activity is very low during the early part of the light phase (in contrast to female rats that have higher HPA activity throughout the day). This provides us with a time window in which we can manipulate the system without interference from endogenous activity. In some studies, we will use both male and female rats, since the dynamic activity of the HPA axis in this species displays sexual dimorphism (e.g. corticosterone pulses have greater amplitude in females than in males). All studies will be performed in adult rats, with some studies performed in older animals since ageing has been associated with dysfunction of the HPA axis.

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

Whilst some experiments will be carried out using tissue collected from animals or in animals under terminal anaesthesia, most experiments will be carried out in awake animals. A large proportion of these will undergo a 45-60 min surgery under general

anaesthesia for intra-cranial infusion: a small hole will be drilled in the skull, a substance infused into a specific region of the brain/pituitary via a fine needle, and the wound closed. Post-operative pain will be managed with analgesic medication. Some of these animals will be humanely killed so that pituitary/brain tissue can be collected for analysis, whilst others will undergo up to two 60-90 min follow-up surgeries for device implantation and/or vascular cannulation: a small hole will be drilled in the skull and a small device (e.g. optical fibre) implanted in the brain/pituitary and secured to the skull with dental cement; a vascular cannula will be implanted and tunnelled through a flexible tethering system designed to allow the animal to freely move around its home cage. Wounds will be closed, and post-operative pain managed with analgesic medication. Tethered animals will be housed individually but alongside other animals whenever possible; the maximum length of time an animal will be continuously tethered is 2 weeks. Once the animal has fully recovered, recording from the implanted device and/or blood sampling will take place. During experiments, animals may be administered substances that affect HPA activity and exposed to mild stressors (e.g. white noise or restraint); these stressors evoke an immediate and robust stress response from the HPA axis, but do not result in overt and lasting signs of distress. Animals will typically be maintained for 2-6 weeks following the initial surgery, and occasionally up to 16 weeks where longitudinal monitoring is necessary.

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

The primary adverse effects are:

Pain
Following surgery, animals are expected to experience some discomfort and mild-moderate pain for a few days, which will be managed with appropriate analgesic medication.

Weight loss
Due to a reduction in food intake following surgery under general anaesthesia, animals are expected to transiently lose a small amount of weight or slow down slightly in their weight gain rate. This will be managed through regular monitoring of animals' weight and providing an appropriate diet.

Stress
Animals are expected to experience a brief period of mild stress when connecting an implanted device to recording equipment; this will be minimised by gradually habituating animals to the equipment in the days leading up to an experiment.

Tethering of animals during recording or blood sampling experiments is also a potential source of mild stress; this will be minimised by selecting lightweight and flexible tethering systems that enable animals to move freely around the home cage.

Animals are expected to remain calm and display normal behaviour when appropriately tethered.

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

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

Rats 60% mild, 40% moderate

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

- Killed
- Used in other projects

Replacement

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

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

In this project, experiments will be performed in the rat. In vitro techniques using primary tissue culture will play an important role in helping to achieve the objectives of this programme of work. However, such methods cannot adequately account for the complex interactions between multiple components of the HPA axis; interactions that we know are critical for the functional oscillating dynamics of the system. Thus, studying the activity of the system as whole, within the living organism, is essential.

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

Where possible, we will integrate mathematical modelling into our experimental programme by using our mathematical models of the pituitary corticotroph cell signalling pathway and pituitary-adrenal system to make predictions about system activity that can be tested experimentally.

Why were they not suitable?

Whilst our mathematical models are valuable in many ways, the predictions they make are not always reliable since the models lack much of the complexity of the real biological system; this means that the predictions they make about system activity must always be confirmed/refuted experimentally.

Moreover, development of these mathematical models, as well as their parameterisation, relies critically on experimental data: our approach will be to use our experimental in an iterative manner to refine and improve our mathematical models of the system, and in turn generate more reliable theoretical hypotheses to be tested experimentally.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 this project is based on a combination of statistical modelling that provides an estimate of appropriate group sizes, extensive prior experience with the various experimental designs, and information on sample sizes that have been required in closely related experiments published in the scientific literature.

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

- To reduce the number of animals required, we took the following steps at the stage of experimental design:
- Performed power analysis calculations, where appropriate, to determine optimal sample sizes.
- Decided on appropriate statistical methods to use to analyse experimental data.
- Designed in vitro experiments, where possible, to optimise treatments (e.g. substance effects on cell function) or generate hypotheses prior to testing in vivo.
- Designed experiments in a way that maximises information gained from the fewest number of animals possible; for example, using the same blood sample to measure several hormones; or using explanted tissue from one animal to measure the expression and activity of several genes and proteins.
- Designed experiments using GA Cre-driver and reporter rats in order to obtain data on specific cells/population types in an efficient manner.
- Planned appropriate experimental controls to ensure that reliable conclusions can be drawn when performing statistical analysis of the data. For example, wild type littermate controls bred under the same conditions as GAAs will be used as control comparisons, where possible.

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

- To further reduce the number of animals required, we will:
- Use mathematical modelling to create testable hypotheses; and only test these predictions experimentally when our mathematical models suggest they are robust (i.e. likely to be observed in the presence of small amounts of variability or noise in the system).
- Carry out longitudinal studies, where appropriate, so that an animal can act as its own control.
- Perform pilot studies, where necessary, before carrying out more involved exploratory or confirmatory experiments. An example would be a time course experiment where animals are killed for tissue collection at various times after a treatment; here we would first carry out this experiment in a small number of animals to assess the most important time points to take forward.
- Produce a ‘study protocol’ for each experiment, which will include the objective(s), a brief description of the experimental design, a description of how the data will be presented, and an outline of the statistical analysis that will be carried out.
- We will minimise inter-animal variability by conducting experiments in as controlled a manner as possible; for example, we will:
- Minimise handling of the animals during an experiment by using implanted cannula(s) for blood sampling and substance administration.
- Minimise environmental noise during the experiments that could activate the HPA axis and thereby invalidate data collected.
- Minimise environmental stressors (e.g. the presence experimenters) during the

experiments by remotely operating blood sampling and substance infusion systems from outside the procedure room, where appropriate.
- Use animals of a similar age and weight, and purchase them from the same supplier, for the duration of each study.
- Process blood samples and tissues simultaneously to reduce inter-variability in the assays used.
- Implant neurophysiological devices and guide cannulas using stereotaxic technique to ensure consistency and a high level of precision.
- Habituate animals to recording equipment and tethering systems in the days leading up to an 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 rats in this project. The rat is the most widely-used animal for studies into the function and control of the HPA axis, and its HPA axis has been better-characterised than that of any other species. This is due, in part, to the fact that the HPA axis in rats and humans shares many similarities, including a pulsatile mode of hormone secretion. The rat is therefore a valuable model in which to answer questions that cannot, for ethical reasons, be addressed directly in humans. The experimental methods used in this field of research are well tolerated by the rat, and do not typically result in overt signs of distress.

In addition to wild-type rats, genetically-altered (GA) rats will be used in this project to enable us to specifically target certain types of cell. The GA rats we will use have innocuous (mild) phenotypes that do not affect animal welfare or cause any harm or distress to the animals. We do not expect adverse effects to be different from wild type animals; that said, all GA will be carefully monitored for potential unexpected phenotypes.

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

The requirement to collect blood samples means that it would not be possible to carry out these studies in very small non-regulated species such as Drosophila.

Zebrafish have become a valuable model in many areas of biomedical research, but unfortunately, they are not suitable for this project since their HPA axis is very different anatomically to mammals; for example, they lack a median eminence and the portal blood supply that transports hypothalamic hormones to the pituitary.
Moreover, it is not possible to take repeated blood samples in the way that is required to characterise hormone oscillations.

In order to record from and manipulate the pituitary-adrenal system in a highly-controlled manner, some in vivo studies will be performed under terminal general anaesthesia. However, since anaesthesia itself can influence neuronal circuits regulating HPA activity, it

will be necessary to perform the majority of the in vivo experiments in freely-moving animals. Moreover, in some studies, in order to establish the dynamic response of the pituitary-adrenal system to environmental perturbations, we will expose the animals to an acute stressor of mild-moderate severity; this can only be performed in freely-moving animals.

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

Since the HPA axis is activated in response to stress, leading to an increase in circulating glucocorticoids, it is important, not only in terms of animal welfare, but for the success of our experiments, that animals display no signs of distress.

Housing
Animals will be group housed whenever possible. When animals do need to be housed individually, we will use cages that allow the animals to see, hear and smell each other; this will help to minimise any effects of single housing that might occur over the short periods of time involved. Animals will be provided with objects (e.g. wood blocks) to enhance their cage environment.

Surgery
All surgery will be performed using aseptic technique. To prevent hypothermia during surgery, we will use a temperature control system to monitor and regulate the body temperature of the animal. To minimise the number of times an animal undergoes general anaesthesia with recovery, we will combine surgeries, wherever possible (e.g. performing vascular and subcutaneous cannulation under the same general anaesthesia). Animals will be given sufficient time to fully recover between surgeries under general anaesthesia. Surgery that involves injections or implantation of neurophysiological devices or guide cannulas in the brain/pituitary will be performed using stereotaxic technique to ensure a high level of precision and thus avoid damage to neighbouring parts of the brain/pituitary. We will select the smallest diameter of implant possible that still enables us to achieve the scientific aims of the study.

Analgesia
In consultation with the NVS/NACWO, analgesia will be given before, during and after surgery for as long as is necessary to alleviate pain.

Post-operative care
Immediately following surgery, animals will be closely monitored whilst in a cage or recovery tank until they have recovered from the anaesthetic. Animals will then be checked at least 3 times on the day or surgery, at least twice per day for 5 days following surgery, and at least once per day thereafter.

Tethered animals will be checked at least twice per day. We will use the NC3Rs Grimace Scale to aid assessment of pain, as well as a distress/condition scoring sheet. Any concerns about the welfare of an animal will be raised with the NACWO/NVS.

Tethering and head/back-mounted equipment
Animals will be tethered in some experiments and may also carry head/back-mounted equipment (e.g. miniature optical fibre). We will aim to minimise the total time animals are tethered or carrying load, whilst ensuring the scientific goals of the study are achieved. Where possible, animals will be progressively habituated to tethering systems and/or head/back-mounted equipment leading up to an experiment. We will also aim to minimise

the weight of mounted equipment, and where appropriate, use counterbalanced levers to support tethering systems. The chosen tethering materials have undergone extensive testing to maintain lightweight and flexible properties to provide a balance between the experimental needs and the maintenance of normal animal behaviour. Based on our previous experience, animals are expected to display normal behaviour whilst tethered (tethers are designed in such a way that animals can explore the home cage with minimal restriction).

Substance administration
Most substances will be obtained from established and reputable companies that guarantee extremely high purity of reagents. Occasionally, substances may be obtained from collaborators; if required, in this case we will carry out pilot studies using low doses and a small number of animals to establish the potency of the substance. For studies that require the administration of substances associated with adverse effects, we will use the minimum dose and treatment time possible whilst still ensuring that the scientific objectives can be achieved.

Administration of substances via an implanted vascular or subcutaneous cannula, either manually or using an automated infusion pump, alleviates stress on the animals that would otherwise be caused by handling and venepuncture or subcutaneous injection. This is of particular importance when needing to administer a substance repeatedly (e.g., delivering a pulsatile hormone pattern).

Blood sampling
Collection of blood samples via an implanted vascular cannula, either manually using a syringe or using an automated blood sampling (ABS) system, alleviates stress on animals that would otherwise be caused by handling and venepuncture. This is of particular importance when needing to take multiple blood samples at high frequency, which is required to characterise oscillations in hormone concentration. Whenever possible we will use an automated blood sampling (ABS) system, which enables us to perform experiments remotely, thereby minimising stress to the animals that may be caused by the presence of experimenter(s).

Remote monitoring of animals during experiments
To ensure that the presence of the experimenter does not influence the behaviour of the animals, and in turn potentially invalidate data obtained, some experiments will be performed remotely (i.e. from outside the procedure room). In this case, animals will be recorded by a CCTV system.

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

To ensure success, validity and reproducibility of our experimental work, we will plan our studies making use of the PREPARE Guidelines (doi: 10.1177/0023677217724823). We will also follow the ARRIVE Guidelines 2.0 (doi: 10.1371/journal.pbio.3000410) when publishing outputs of this project, which will ensure our results are reported with clarity and in a manner that promotes reproducibility.

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

Throughout this project, we will ensure we remain knowledgeable of the latest 3Rs advancements by regularly checking relevant online resources such as the websites

managed by LASA (https://www.lasa.co.uk/) and the NC3Rs (https://nc3rs.org.uk/).

We will work with the NVS/NACWO to incorporate into our work any new and relevant 3Rs developments. We routinely discuss experimental refinements with our network of collaborators performing similar techniques and will continue to do so throughout the course of this project.

32. Neural function in sensory systems

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Brain function, Senses, Metabolism, Ion channels, Receptors

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

Understand how the brain integrates external and internal sensory information and what goes wrong with disease-associated mutations.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 gather information from the external world through our sensory systems, and crucially we integrate this external information with our internal state, e.g. the sight or smell of food evokes a different experience when we are sated vs hungry. Yet how our internal state interacts and modulates the information arriving from our environment is poorly understood. Insight in this area could be used to modulate the hedonic aspects of food intake with implications for ameliorating eating disorders and the worldwide increase in rates of obesity.

Throughout life, we are continually updating our sensory perception, whether this is improving our ability to distinguish notes of a perfume, a wine, or a concerto. New stimuli can become associated with positive or negative valences. How sensory systems learn these associations is poorly understood as is how obesogenic environments modulate

normal sensory responses to food-related stimuli. This project will provide important insight into these questions.

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

The major outputs of this project will be new information on how sensory and interoceptive signals are integrated by the brain, how this changes with metabolic state and how food-odours are encoded by the brain. This information will also generate scientific publications.

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

As this project addresses fundamental questions in neuroscience and metabolism the main impact of this research will benefit researchers in these communities. Longer term broad economic and societal benefits could be realised from a better understanding of how sensory physiology controls food intake,
e.g., intranasally administered drugs could target components of the circuitry that promotes satiety, reducing food intake and reducing rates of obesity.

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

The outputs of this work will be published in scientific journals, communicated at national and international conferences and via collaborations with other labs.

Species and numbers of animals expected to be used

- Mice: 3650

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 modified mice between 8-24 weeks. Only the genetic tools available in mice enable the objectives of this project to be met and, unlike other non-mammalian vertebrates, the structure of the mouse olfactory system is closely related to the human.

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

Mice may be fasted overnight and receive intraperitoneal injections of non-toxic substances. Typically, mice will undergo surgical procedures to enable imaging of neural activity in the brain and for the majority of mice this will be performed under terminal general anaesthesia. Some mice will have a cranial window implanted during recovery surgery and will subsequently be habituated to head restraint to permit imaging of brain activity in the awake state. Some mice will undergo fear conditioning involving a brief foot shock.

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

Pain/discomfort may result from the recovery surgical procedures, animals recover from surgery quickly, regaining normal behaviour with 1- 2 days, any pain/discomfort is minimised with analgesics and careful postoperative care. Weight loss is likely after overnight fasting but this is transient being rapidly regained after the return of food. Head fixation on a low-resistance treadmill is initially stress- inducing, however this is transient and diminishes after 10-25 mins of habituation spaced over 2-3 days. Foot shocks are likely to induce pain but this is very transient lasting <1s.

Expected severity categories and the 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 will be non-classified (sub threshold) as mice will be killed by schedule 1, 16% of mice will have a severity rating of mild due to overnight fasting and 7% of mice will have a severity of moderate.

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

- Killed
- Used in other projects

Replacement

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

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

It is only possible to study multisensory integration, i.e. how external stimuli are integrated with internal sensory cues, in intact animals.

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

There are no non-animal alternatives for studying sensory perception and its, integration with internal state.

Why were they not suitable?

N/A

Reduction

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

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

The number of animals that will be used in this project are based on the scientific questions that will be asked and the experiments necessary to address them. To estimate the number of animals that we will need to use for breeding, we used our annual return of procedures data based on similar work on a previous PPL.

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 will be minimized in several ways:
The NC3RS experimental design assistant will be considered for planning:
https://www.nc3rs.org.uk/experimental-design-assistant-eda

A majority of planned experiments are paired in nature, this reduces extraneous variability, increases statistical power, and therefore requires the use of fewer animals.

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

We endeavour to maintain efficient breeding strategies and routinely take advice from the NACWO and animal technicians in how to achieve this. Where possible we employ computer simulations which help to guide future experiments. Wherever possible we share tissue with other labs.

Refinement

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

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

We will use genetically modified mice throughout this project. The majority of procedures will not cause any suffering above mild. For some experiments it will be necessary to administer non-harmful substances into the brain, which will require surgery. Careful perioperative care and postoperative monitoring will ensure any suffering is at a minimum. In some experiments, we will need to implant a head restraint and cranial window in order to image the brain during learning. Again careful monitoring and perioperative care ensures that animals recover quickly with minimal suffering. These animal will need to have their head restrained during imaging periods, to minimise the stress this may cause, we ensure animals are free to move their body on a low resistance treadmill, allowing them to walk/run, rest and groom of their own volition. In addition animals are gradually habituated to this apparatus over a time period tailored to each animal.

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

Only the genetic tools available in mice enable the objectives of this project to be met and, unlike other non-mammalian vertebrates (e.g. zebrafish), the structure of the mouse olfactory system is closely related to the human.

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

We will provide careful perioperative care, and post-operative monitoring. Habituation to head restraint will be tailored to each animal to minimise distress. Routine video-monitoring of head-fixed animals during experiments will ensure animals only participate in experiments as long as they are stress free.

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

I will follow the best practices recommended in the recent report on head fixation experiments that was the result of an expert working group convened by the NC3Rs (see below).

Barkus, C., Bergmann, C., Branco, T., Carandini, M., Chadderton, P.T., Galiñanes, G.L., Gilmour, G., Huber, D., Huxter, J.R., Khan, A.G., King, A.J., Maravall, M., O'Mahony, T., Ragan, C.I., Robinson, E.S.J., Schaefer, A.T., Schultz, S.R., Sengpiel, F. & Prescott, M.J. (2022) Refinements to rodent head fixation and fluid/food control for neuroscience. J Neurosci Methods, 381, 109705.

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

We receive newsletters from the NC3Rs, we rely on the website to find important information regarding ways to reduce, refine and replace (https://www.nc3rs.org.uk/).

I also supervise a NC3Rs funded PhD studentship that attends NC3Rs events and conference and who keeps the lab abreast of recent developments.

33. Understanding neuropathology and neurodegeneration in dementia

Project duration

5 years 0 months

Project purpose

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

Key words

Neurodegeneration, Dementia, Alzheimer's disease, Parkinson's disease, neuropathology

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 causes and consequences of neuropathology in the diseases that cause 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 the number one killer in the UK. The diseases that cause dementia have no way of being prevented, slowed or cured. Understanding the mechanisms underlying these diseases is vital to developing new treatments.

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

This fundamental research will increase our knowledge of how specific proteins that accumulate in Alzheimer's disease, Parkinson's disease and other neurodegenerative conditions lead to changes in brain cells.

Outputs from this work will include publications in peer-reviewed journals, presentations at research conferences and to the public through engagement initiatives through the university/charities.

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

Our research aims to understand how the build-up of specific proteins leads to the development and changes in brain cells in neurodegenerative diseases such as Alzheimer's disease or Parkinson's disease. This work will initially benefit other research groups and scientists working in this field. Beyond this, the fundamental knowledge gained may have the potential to be used to develop therapeutics for the people living with these diseases that cannot be currently prevented, slowed or cured.

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

The results and potential tools from this research will be shared at scientific conferences and open access through pre-prints, and then in open access peer reviewed journals. We will also share negative results to maximise outputs.

Species and numbers of animals expected to be used.

- Mice: 5000

Predicted harms

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

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

We are using mice because they are the lowest order vertebrates with brains that are similar to humans. There are also mice available that are genetically changed allowing us to study diseases such as Alzheimer's disease and Parkinson's disease. We will use brain tissue taken from neonatal mice as we have developed ways to keep this tissue alive in a dish, preventing the need to age the animal. For other experiments, we will use adult and aged mice, as they are highly relevant to study neurodegenerative diseases that increase with age.

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

Some mice with specific genetic traits will be bred to maintain the particular colonies and produce experimental animals.

In over half of the experiments brain tissue will be obtained from early postnatal mice that have been humanely killed.

In the remaining experiments, mice may receive anaesthesia after birth to have substances delivered into their brains. These substances mainly supply non-infectious viruses which will allow us to understand how certain genes linked to dementia change the brain. After this, these mice may undergo non-invasive behavioural testing that would cause no harm or distress or receive other substance injections (typically potential therapeutic compounds) intended to reduce the dementia-like brain changes. These will then be humanely killed in order to study their tissues.

In approximately <5% of experiments, mice will be aged beyond 12 months typically to 18 months old. This is because ageing is the biggest risk factor for dementia and will allow us to study how ageing also modulates the brain in this context. These mice will be humanely sacrificed at the end.

When there could be pain or distress associated with the intervention, mice will receive pain relief and will be closely monitored and treated with supported food and caring / nursing approaches (e.g. palatable enriched feeding; supportive enrichment and caring particularly for pups and /or aged animals).

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

The majority of mice will undergo subthreshold or mild experiences, typically for breeding, or ear clips for identification that are brief in nature, or injection of substances/behavioural testing that are not harmful/distressful.

A small proportion of animals may approach a moderate severity, this is due to the ageing process or acceleration of the ageing process to study Alzheimer's/Parkinson's disease. These mice will be carefully monitored for development of signs of distress or certain discomforts.

At any stage if mice show signs of ill health, distress or suffering that are not improved or resolved with supportive treatments (e.g. analgesia treatment, supportive food and husbandry to minimise distress) they will be humanely killed. Humane endpoints will be applied to ensure the animals do not sustain moderate persisting distress.

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

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

70% of mice will experience a severity category 'Mild' or 'Sub-threshold 30% of mice will experience a severity category '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?

Mice will be used in this project as they have a very similar nervous system to humans. To study conditions like Alzheimer's disease and Parkinson's disease requires us to observe the complex interactions between the brain and whole body. It is hard to mimic all of this ex vivo, so some whole animal in vivo experimentation is required to increase our knowledge of these diseases.

Where we use ex vivo brain slice tissues, we can mimic some of the changes of an intact brain where certain parameters are well preserved. However, where we need to see the complex interactions of the brain and body, this cannot be fully studied here and whole animals are used.

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

Induced pluripotent stem cells (iPSC) or brain organoids from humans Post-mortem brain tissue.

Why were they not suitable?

iPSC or brain organoid systems do not allow the study of interactions between the brain and whole body which are important for our whole understanding of Alzheimer's disease and Parkinson's disease, nor do they model the intact brain.

The use of human post-mortem tissue only provides one snapshot at the end of disease whilst the brain is undergoing organ failure. It does not allow us to see the events preceding and building up to this which will be the most useful timepoints to start to treat the disease. We need to observe these events 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?

This estimate is based on previous experimental experience as well as statistical techniques to estimate the number of experimental animals required based on expected effect sizes from the literature and own past work.

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

We have used the NC3R's Experimental Design Assistant and employed statistical power calculations using analysis software.

We will also use efficient mouse breeding strategies to minimise the number of animals

used.

Using ex vivo brain sections from neonates also reduces the need to age larger colonies of mice and reduces some of the in vivo work. We also use a procedure to accelerate dementia in some animals that will reduce the numbers of animals required for these studies as it reduces variability and increases reproducibility for the protein build-up we are studying.

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 prevent surplus animals. The experimental value of each mouse will be optimized by maximising the number of measurements taken from each animal, and tissue will be shared when possible, to reduce the need for mouse use by other groups.

Pilot studies will be undertaken to ensure the correct time course and experimental paradigms for our experimental purposes before larger scale 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.

Mice have been selected for this work as they are the least sentient species that have an intact nervous system similar to that in humans. Mice are also chosen for this work as they have been genetically changed to allow us to see features of Alzheimer's disease or Parkinson's disease. These genetic changes can lead to neurodegeneration and this can be accelerated using an injection of a substance but the symptoms only develop after a period of time. This enables us to closely monitor the mice, and humanely sacrifice any that develop distressing symptoms, to minimize harm.

We also plan to extract tissue from mice for ex vivo approaches. These experiments allow us to test certain factors before testing them in vivo, which will help to reduce the number of animals undergoing the in vivo paradigm and that may suffer as a result.

For brain injections of substances where light anaesthesia is used - mice will undergo suitable post- procedural care and monitoring to minimise pain. We will continue to monitor resources that may identify further refinements to minimise animal suffering including optimisation of anaesthesia/analgesia regimens.

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

Mice are the least sentient beings that have a nervous system similar enough to humans and that can be genetically changed to model human neurodegenerative disease. These

transgenic models are well characterised.

Animals at a more immature life stage are used for some of our experiments but ultimately neurodegeneration occurs in adults and aged animals so these need to be examined too.

Some of our experiments are done in animals that have been euthanised, but to observe long-term disease relevant changes and the complex interaction between brain and body we need to perform some chronic studies.

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

Animals undergo regular health monitoring provided by the animal technical staff and PIL holders. We use appropriate anaesthetic/analgesic regimens (including pre- and post-operative analgesia) to minimise pain and will refine these with advice from the NVS to ensure that we are using the best possible option given our experiments.
Specifically to this project, genetically modified animals that can show signs of neurodegeneration are not kept alive longer than is absolutely necessary for the proposed experiments.

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

We will follow the ARRIVE, PREPARE, and FELASA guidelines for all experiments conducted.

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

We will follow any advances through the NC3Rs website, closely follow the scientific literature relevant to the project, and through contact with our animal care providers.

34. Nutritional and environmental interventions to improve aquaculture

Project duration

5 years 0 months

Project purpose

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

Nutrition, Physiology, Fish, Diet, Environment

Animal types	Life stages
Zebra fish (Danio rerio) Embryo,	juvenile, adult
Salmon (Salmo salar) Embryo,	juvenile, adult
Rainbow Trout (Oncorhynchus mykiss)	Embryo, juvenile, adult
Pangassius (Striped catfish), Oreochromis (Tilapia) and Clarius(African catfish) species	embryo, with relevant commercial value for aquaculture. 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 principal aim of this programme of work will be to advance our understanding of the physiological, biochemical, molecular and immune responses of fish and how changes in environment (e.g. temperature, oxygenation etc.) and diet (e.g. specifications, ration, raw

material choice etc.) influence these responses when applied throughout a fish's lifecycle.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 driven by the adaptive needs of the aquaculture industry and associated stakeholders and their need to define responses to changes in nutrient supply and/or raw material use with various species. Furthermore, there is a need to understand at a deeper level the impact of environmental interactions in aquaculture systems and their influence upon nutritional requirements. Aquaculture production systems may be entirely closed or semi-closed from the external environment or open- systems that directly interact with the external environment. This work aims to understand such interactions at the level of farmed population and the abiotic impacts from the environment that for example may represent the impact of differential dissolved gases in closed recirculating systems or thermal variation in open/cage production systems upon the nutritional requirements of the farmed population. The nature of this work responds to emergent needs as commercial aquaculture explores a range of primary materials, for example black soldier fly meals, that may contribute to the sustainability across global aquaculture activities.

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

Outputs are expected both for research/academic use and those with direct commercial impact. For outputs with commercial potential addressing any area of nutritional improvement that results from this work further industry-led development is expected. This may include novel diet formulations for different species at distinct life stages, the inclusion of novel diet components into standard diets as replacements for less sustainable ingredients or the inclusion into commercial diets of novel compounds that exert beneficial effects upon fish health and welfare.

For academic/research outputs we will implement a dissemination strategy designed to maximise timely sharing and publication of research with consent from industry partners. Given the current public and commercial interest, we believe it will be a realistic aim to publish the key results in high-impact peer-reviewed journal such as Reviews in Aquaculture, and further disseminate applicability through more discipline-specific journals (e.g. Frontiers in Aquaculture). Drafted research manuscripts will be shared on pre-print portals such as bioRxiv.org before final publication in open access peer-reviewed journals. All sequence data will be shared using public data portals, such as NCBI or EMBL-EBIs European Nucleotide Archive. When relevant we will engage a communication company (e.g Scientifica) to produce video material and targeted messages about the project and project findings.

This content will be distributed through all the relevant communication channels available as well as through social media. Information about the project and project findings will also be published as popular scientific summary articles in relevant aquaculture news media such as Kyst.no and Intrafish.

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

There will be clear benefits from undertaking this project to humans, animals and the

environment. As a result of this project there will be improved animal health and welfare (benefit to the animals). This will further contribute to improved industry performance by development and application of novel diets and nutritional management strategies driving a reduction in poor-health associated losses (benefit to humans) and also improved industry sustainability (benefit to humans and environment). Ultimately the outcomes of this project will also allow for a reduced environmental impact associated with aquaculture (benefit to environment) by underpinning a more efficient use of resources and an ability to include more sustainable raw materials in feeds.

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

As most of the work is carried out in collaboration with commercial entities targeted workshops and short articles in popular scientific press have proven to excellent vehicles to maximise dissemination outputs.

Species and numbers of animals expected to be used.

- Zebra fish (Danio rerio): 5000
- Rainbow Trout (Oncorhynchus mykiss): 5000 • Other fish: No answer provided
- Salmon (Salmo salar): 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.

A range of representative freshwater and marine species that are currently under commercial production are expected to be used, including but not exclusive to Atlantic salmon (Salmo salar), tilapia (Oreochromis niloticus), Clarias catfish (Clarias gariepinus), zebrafish (Danio rerio) and rainbow trout (Oncorhynchus mykiss). Life stage choice will be determined by the experimental framework applied for example first feeding or weaning. As nutrition formulations change throughout production cycles and are specific to life stages we expect to work across all life stages.

The Atlantic salmon (Salmo salar) is the most significant aquaculture species in the UK with a value of >£1Billion to the UK economy. The Atlantic salmon has a complex anadromous lifecycle consisting of both freshwater and seawater life stages. The rainbow trout (Oncorhynchus mykiss) is of significant value to the UK and has importance worldwide in terms of aquaculture production. The rainbow trout can be produced in both freshwater and freshwater-seawater combined systems therefore is the most versatile salmonid species in aquaculture. Tilapia (Oreochromis niloticus) is the second most grown fish across our planet and is present in all continents. Tilapia have a critical role in global food security and are farmed in both intensive and extensive production systems under diverse environmental conditions and in many countries. Clarias catfish (Clarias gariepinus) is the dominant aquaculture species in Nigeria with over 160000 tonnes output per year and 2 million tonnes global output with a future projection of significant increases in production due to its environmental resilience in terms of freshwater quality and water temperature. The zebrafish (Danio rerio) is a widely used model species in the biological and biomedical sciences used in cutting edge research. The zebrafish is an excellent model for nutritional and health studies in fish in terms of basic research in fish and is

supported with excellent genomic and biological resources. On the other hand, nutritional management of zebrafish is poorly developed and its development through for example novel tailored diets has significant potential to improve health and welfare of zebrafish in current production systems.

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

This project licence involves procedures which are all categorised as “mild” or “moderate” in terms of effects. The techniques applied can be used across all fish species that are currently grown in aquaculture production systems including both intensive (closed recirculation) and extensive (open) systems. Most of the protocols described in this project licence involve techniques that are used in routine fish management (e.g. alteration of diet, temperature, salinity, oxygenation, weight-length assessment, faecal stripping). A typical experiment will consist of novel diet formulations where critical diet factors are added at different inclusion levels and fish will be fed over specific time periods and/or production stages and performance measured. Performance may include growth rates, condition factors, weight-length variation, operational welfare indicators, health measures. The duration of experimentation is dependent upon the lifecycle of the fish species and the production methods. A minimum duration such as a first feeding/weaning experiment may be 15-21 days whereas a seawater diet application may last >9 months.

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

Few adverse effects from the allocation of a specific dietary or environmental treatments are anticipated across the duration of experimentation which is typically between 3-12 weeks. Weight loss due to the experimental diets with and without environmental manipulations are closely monitored with specific thresholds identified through a traffic light system. During the instigation of any treatment the fish will be closely observed with any moribund fish being euthanised.

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

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

The project involves procedures that are mostly categorised as “mild”, though the severity limit to “moderate” based on the potential for growth retardation of >15%, but <25% of expected growth associated with the need for some negative control treatments to definitively demonstrate nutrient limitations in diets where responses to “adequate” levels and above are also being assessed. This may involve up to 25% of the animals within a specific experimental design.

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

- Killed

Replacement

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

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

The mechanisms we are interested in are the outcomes of the interaction of various nutritional and environmental conditions on the complete physiological response and no single isolated cell-based model has yet proven effective in replicating the outcomes achieved in a live animal model. The inclusion of environmental variables in this project licence as an additional layer of complexity on top of the nutritional variables also makes it difficult to apply non-animal experimentation and produce meaningful results. Additionally, the applied evaluation of nutritional variables of relevance to the commercial sector requires the production of data that allows for cost-benefit analyses that relate to production situations (i.e., animal performance traits). Additionally, the direct extension of experimental outcomes to commercial practices requires the use of live animal studies that are accepted by industry as the most reliable basis for underpinning evidence for change. Although most of the work in this project licence requires animal-based experimentation, in certain circumstances isolated discrete tissues (e.g. a liver cell culture) may be utilised.

Where possible, non-invasive monitoring of fish behaviour will be used to further assess the welfare of the animals in response to variations in diet and environment.

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

A variety of resources (e.g., literature and scientific networks) have been used to identify potential alternate models to the use of animals in these studies. Alternatives such as in vitro digestibility models and cell cultures have been considered but are deemed insufficient alone to replace the biological effects of a live animal model.

Why were they not suitable?

Due to the need to provide data relevant to commercial aquaculture systems. The physiological responses we are investigating in this program of work and the associated biological responses (e.g. growth) need experimentation on animals. In vitro digestibility models address dynamics of the gut- associated microbiome in terms of interactions with diet and have limited application toward understanding fish nutrition (nutrient uptake) however have potential to unravel bacterial contributions to digestion in the gastrointestinal 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 numbers of animals used in each experiment will be defined by an appropriate minimum sample size relevant to the minimum biological effect and the inherent variance associated with the primary variable being examined. For example, typically three replicate tanks will be used for each treatment in an experiment. If the variable being examined is smaller than 10% effect size, a greater number of replicate tanks may be required, though

power analysis a priori can help define this. Additionally, a minimum threshold number of fish (e.g. 20+) is required in each tank to induce normal feeding behaviour. This tends to be species specific (e.g. salmonids >20 fish, tilapia >30 fish) due to the formation of social hierarchies, but if fish are to be serially sampled throughout the course of anexperiment then the final number of fish in the tank prior to experiment completion also needs to be considered. Therefore, in situations where there is planned to be sampling over the time-course of an experiment to examine rates of responses to treatments (e.g. sampling at month 1, 2 and 3), additional fish will need to be included at the outset to ensure at least 20 fish are maintained within the tank throughout the course of the experiment (e.g. including 40 fish in a tank with sampling of 10 fish at month 1, 10 fish at month 2 and 10 fish at the end of a three month trial). The total number of animals proposed over the term of this project licence is an approximation based on conducting two trials of 24 tanks x 40 fish (960 fish) for each of the four species, each year of the five years of this licence (approximately 40,000 fish). However, we anticipate a greater number trials with some species (e.g.Atlantic salmon) and less frequent trials with other species (e.g. rainbow trout).

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

The numbers of animals required for each experiment will be defined by the use of appropriate statistical pre-tests and design software (e.g. Experimental Design Assistant; https://www.nc3rs.org.uk/our-portfolio/experimental-design-assistant-eda) to define the minimum potential number of tanks of fish required, in conjunction with any logistical constraints due to behaviour of fish within a tank (e.g.number of fish within a tank to avoid social hierarchy formation) and the required sample size (e.g.amount of faeces) to undertake a robust analysis of the samples collected.

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

Animals used will be sourced from commercial breeding programmes therefore from proven performance backgrounds. Pilot studies are incorporated into protocol 1 where required e.g. palatability studies and where not possible we apply a traffic light evaluation system for the managment of trial progression. In terms of sample management a significant biobank of samples is available and will be built upon in order to reduce the numbers of experimental animals that are being used. For example for the development of species-specific molecular tools archived sample material is critical to the development of projects and the use of these materials supported with bioinformatic mining of genomic resources reduces the need for additional experimental 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.

Work will be carried out on commercially important fish species for aquaculture. Due to the diversity of the species, farming systems and environments used in aquaculture it is important to target a range of species in this project licence application to ensure project objectives can be attained for the desired relevant species and industry sector. Throughout the work all fish will be housed in conditions that comply with the welfare standards of the target species of interest and monitored regulalry by the PILh and the NACWOs with oversight from the PPLh and the NVS.

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

The animal models used are based upon commercial applications for food production and therefore the use of different life stages are essential to the experimental work.

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

To further refine these procedures, built upon >10 years of reflection and refinement of husbandry and experimental procedures, fish will be housed in facilities that are constantly monitored for temperature and oxygen, allowing further refinement of critical environmental constraints. We have developed a series of non-invasive monitoring methods based upon behavioural analyses for example during first feeding experimentation. We will apply these approaches throughout our experimentation where possible.

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

Scientific literature, RSPCA recommendations, commercial certifications relevant to aquaculture for example GAA and in house discussion and publications of best practise. Our establishment represents a significant community of scientists and research students involved in these activities and is a global center of reference for fish welfare.

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

We will follow current practise and advances in practise by regularly consulting and contributing to the NC3Rs platform. We have current activities funded by the NC3Rs that serve as access to the platform and regular meetings with other scientists involved in 3Rs research provide opportunity for discussion and sharing of recent advances in this area. Furthermore, regular conference attendance for example Aquaculture UK where aquaculture stakeholders from research and industry are in attendance presents opportunity to present and discuss 3Rs context in commercial aquaculture operations.

35. Characterisation and gene-drug interactions of neural crest cell lineages associated with cancers and genetic disorders

Project duration

5 years 0 months

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

Key words

Cancer, Stem Cells, Imaging, Genetics, Drug-Development

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The purpose of this project is to understand what cell types contribute to cancers, such as melanoma, and other disorders and to find treatments which target those 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?

A striking discovery in the past decade has been that cancers are made up of different cell types, and that some of these cells are more dangerous than others. Our objective is to

understand how stem cell populations, such as neural crest cells and the cells derived from neural crest, can contribute to cancer development when they become damaged. Stem cells are the body's master cells that give rise to many cell types and tissues. We have evidence that one cancer stem cell type is responsible for initiating new tumors in the body, and may contribute to resistance to therapy. Based on our findings, we are designing new therapies to target these cells in cancer. We are specifically interested in the skin cancer, melanoma, and developing new drugs that might be useful in treating melanoma.

Melanoma is the most deadly form of skin cancer, and rates are rapidly rising in Scotland. If caught early, melanoma can be cured by surgery. However, once the disease spreads most patients still die of the disease. Our work is based on identifying new drugs to target melanoma, and to understand how the cells within a tumor contribute to disease progression and respond to therapy.

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

Helping to understand disorders and supporting the development of methods to manage and treat human disease, such as skin cancer.

Expected benefits:

1. New understanding of neural crest cell and melanocyte stem cell biology. These studies are worthwhile because melanocytes are the pigment producing cells in our body, and defects in these cells can lead to pigmentation disorders and cancer. In the short-term, we expect this new insight to be used by our group and other researchers. However, we interact closely with clinical and patient groups, and take a long-term view that the work may lead to new hypotheses and insight into human pigmentation and disease.

2. New understanding of melanoma biology. We expect to identify new genetics and melanoma subpopulation dynamics that contribute melanoma progression, regression and relapse. Many patients respond to BRAF and MEK inhibitors but the cancers often recur. Our zebrafish models are providing new insight into melanoma recurrence, and we expect to be able to directly test the number of cells that remain following melanoma regression, understand which ones contribute to recurrence and target these with chemicals/drug-leads. This work is basic science, but we aim to perform follow up studies when possible in human melanoma tissue to link it to patient disease progression and outcome.

3. Small molecule leads for drug development. An important part of our work is the identification of targetable pathways in melanoma and other cancers. We have identified small molecules that target melanocyte and melanoma cell populations and these present new opportunities for academic drug development. We are currently collaborating with clinical researchers to establish the preclinical studies to move these studies forward to patient populations . Further, we have recently established a new collaboration with a pharmaceutical company to further develop the translatability of our work.

At the end of this 5-year PPL programme, we aim to have identified novel genetic and drug-leads that regulate the melanocyte stem cell lineage in melanoma. During the course of this work, we will be working closely with NHS pathologists and clinical scientists to test the translational relevance of our work in human cancer cell lines and patient samples. Thus, while our work is rooted in basic science, our model has important translational implications for patients.

Expected benefits will be shared with the wider research community through published papers and at local, national and international scientific meetings. For example, we attend yearly zebrafish and cancer conferences , Melanoma Research Alliance, Society for Melanoma Research (International), Zebrafish Disease Models Society (International)). The findings will be also shared with non-scientific community through public engagement events locally (e.g. Science Festival, Open Doors Day) and at national level (e.g. radio programmes, podcasts, public news outlets).

The scientific output from the work performed under the previous similar licence has been and will continue to be published in peer reviewed, high impact, leading journals.

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

In the longer term, people suffering from cancers or genetic disorders may benefit from medical advances informed by this work. Human cancers and genetic disorders are varied and complex and will impact almost every human being directly or indirectly through relatives and friends. The real costs are, realistically, incalculable but enormous. Understanding the underlying genetics and cell types involved in a model vertebrate, the zebrafish, is a major step towards developing treatments for these disorders.

We expect to learn about the biology of melanoma and other cancers, and then apply this knowledge to the development of new therapeutics. We work closely with clinical collaborators to test our findings in the clinical context, and some of our work has been part of the basis for a clinical trial which may help improve cancer treatments for humans.

In the shorter term, the discoveries we make and share through peer reviewed publications and the techniques we develop will help advance the scientific field.

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

Expected benefits will be shared with the wider research community through published papers and at local, national and international scientific meetings. For example, we attend yearly zebrafish and cancer conferences , Melanoma Research Alliance, Society for Melanoma Research (International), Zebrafish Disease Models Society (International)). The findings will be also shared with non-scientific community through public engagement events locally (e.g. Science Festival, Open Doors Day) and at national level (e.g. radio programmes, podcasts, public news outlets).

When we create new genetically modified zebrafish, we will maximise the utility of these lines by sharing them widely, as appropriate, to researchers around the world. This is supported by the facility team who have experience with embryo and animal export.

Researchers at our institute are encouraged to submit findings to bioRxiv (https://www.biorxiv.org/) which will make their findings available to everyone, with or without publication in a peer reviewed journal.

Knowledge gained and improvements devised to scientific techniques on this PPL will be shared with the local, national and international scientific community through regular local meetings organised by researchers and attendance of conferences.

Ongoing improvements to protocols on this licence will be used to inform other licences within the university, present and future, through the university vets and the Animal Welfare Ethical Review Board.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 44,500

Predicted harms

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

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

The zebrafish is one of the fastest growing model organisms in terms of exponentially growing numbers of research articles, funded projects and new discoveries associated with the use of zebrafish for studying development, human diseases and screening for new drugs. Thanks to the development of novel technologies, the range of zebrafish research is constantly expanding with new tools synergistically enhancing traditional techniques.

The zebrafish is a unique vertebrate model system that can provide valuable insight into human genetics and biology due to multiple factors:

- The zebrafish is a small vertebrate whose genome has been sequenced.

- The genomes of zebrafish and humans share significant similarity. This similarity can be found at the levels of cells, tissues and organs.

- Rapid embryonic development occurs such that by 48 hours post fertilisation all major organs have developed and are functional. This development naturally takes place externally so no invasive procedures are performed on the parent animals.

- Due to this rapid development, many studies can be carried out on the majority of organs before the embryo is classified as a protected animal thus greatly contributing to Reduction.

- Even before 5 days old, the larvae exhibit stereotyped behaviours which can be studied.

- External development and transparency of the embryo allows longitudinal studies (multiple time points from one animal) which, in other models, would require animals to be sacrificed for each time point.

- The transparency of the embryo synergises with the vast genetic toolbox of the zebrafish to allow expression patterns to be followed in vivo.

- Embryos are freely permeable to soluble drugs and vital dye staining,

- Large clutch sizes of large (1mm diameter), robust eggs are laid daily on cue (light) resulting in synchronised cell development of hundreds of embryos.

- These robust embryos are accessible and readily amenable to well-established genetic manipulation, with no harm to the mother.

- A wealth of knowledge (databases and resources) already exists for zebrafish (e.g. www.zfin.org; www.ensembl.org/Danio_rerio/)

- For a vertebrate housing facility, required space and costs are modest, thus supporting cost effective research.

For these reasons, use of zebrafish as a model organism in the UK is rapidly increasing.

We focus on genetics and how cells interact. Genetics has an impact on almost all aspects of human biology and, increasingly, medicine. While the study of human populations and patients can inform us about the role of genes, a full understanding of gene function and how these genes interact with environmental factors and drugs still relies on the study of model organisms.

The vast majority of work will be done with embryonic/larval zebrafish which are less than 5 days post fertilisation. At this stage they do not have the status of protected animals as their capacity to suffer is less than that of more developed animals. For some studies, such as how cancer responds to drugs, we have to use adult animals as the cancer in an adult animal is more comparable to human cancers.

When creating new genetic modifications, in the vast majority of cases the alterations will made at the 1 cell stage (immediately after fertilisation of the egg). At this stage the fertilised egg is incapable of suffering.

For the minority of experiments that may cause suffering to fish, a living vertebrate animal (comparable to humans genetically and in terms of organ development) is essential to

study disorders, such as cancer, which impact humans through complex interactions within the body. Any alternatives to study human genetic disorders in a vertebrate model would be animals which are arguably more sentient and therefore the alternatives would involve increased potential for suffering.

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

The majority of animals on this licence will not have any procedures performed on them. They will be kept in our state of the art aquariums to produce fertilised eggs by natural spawning which will be used in experiments.

A minority of animals will have a small piece of fin removed by scalpel under anaesthetic which will grow back fully within 2 weeks.

A minority of animals may be non-invasively imaged by anaesthetising them for brief photographing under a microscope or other imaging modalities.

A minority of animals may develop cancer over the course of several months.

A small minority of animals that develop cancer will be treated with drugs which may regress the cancer.

A small minority of animals may be treated to activate their modified genes by briefly heating their water by about 10C or immersing them in a dilute concentration of a commonly used drug which is not harmful at these concentrations.

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

There are two aspects of our work that may cause moderate severity levels. First, we are developing zebrafish that have mutations or will be transplanted with mutated cells, and some of these fish develop cancer. Most of the fish are otherwise healthy, in spite of the tumour growth on the skin.

Over the course of our experiments, the zebrafish will be exposed to some procedures that may be harmful, including anaesthesia, cell transplantation and electroporation. These procedures are well established in the scientific community, and we will work closely with our zebrafish facility staff and University vets to minimise suffering for the zebrafish. Adverse reactions can include inflammation, bleeding and tissue damage, and we will carefully monitor zebrafish for signs of stress or distress (such as difficulty swimming or rapid gill movements). We minimize the development of these adverse effects by training our new staff in all techniques, using state-of-the-art equipment and by performing techniques under anaesthesia when appropriate. Any fish that show adverse reactions that cannot be controlled or reversed will be killed by a humane method to immediately end suffering.

Second, we will be treating fish with anti-cancer agents, and the fish may develop adverse effects to the drug. This can include skin irritation, gill and skin inflammation and unexpected off-target effects. We will limit the potential of these effects by using the lowest concentration of drug we can. We determine the drug concentrations by first testing the drugs on human cells in a dish (that are not animals and do not suffer), and then by testing on zebrafish embryos before they are protected as animals (before 5 days of development). This provides a reasonable dose range to test drugs on adult fish that will have maximal target engagement while having minimal off-target effects. Any fish that is no longer able to swim well, or has evidence of suffering, or additional unexpected signs of disease or illness will be killed in a humane method.

Most fish will not experience any adverse effects from the changes made to their genes. As some of the genes changed are related to human disorders, a minority of fish may have problems which relate to the function of the gene. Fish with genetic changes related to skin cancer will have a higher rate of developing skin cancer.

The majority of fish will experience no adverse effects.

A minority will experience minor, temporary discomfort, such as cutting off a small piece of a fin under anaesthetic (which then grows back naturally).

A small minority (likely to be less than 5%) will experience medium adverse effects, such as developing skin cancer. Any zebrafish which is approaching the limit of allowed severity will be humanely euthanised before the limit is exceeded.

All zebrafish will be humanely euthanised at the end of the experiment or when they reach their maximum healthy lifespan.

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

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

Based on previous experience, 68% of animals will experience subthreshold severity. 27% will experience mild severity. 4% will experience moderate severity.

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

- Killed
- Used in other projects

Replacement

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

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

We need to use animals because we are unable to address our questions in any non-animal system and have it directly relevant to human disease. We need to study the function of the genetic mutations and the potential drugs in the context of a living organism. Animals are required because there is no non-animal system that can provide us with the insight into how these genes work within the context of a living vertebrate system.

We try to only use zebrafish after first learning as much as we can using computer models, cells in dishes and simpler organisms.

Most of the experiments will be carried out on zebrafish larvae that are less than 5 days old and not considered developed enough to be protected animals.

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

Sometimes, we can address some of our scientific questions using cell cultures, or other systems such as yeast or in vitro, and we have the experimental set up in our lab to do these experiments when we can.

Whenever possible we use zebrafish embryos at early stages in development when they are not considered developed enough to be protected animals.

Why were they not suitable?

Cancers and most genetic disorders are dependent on complex interactions between genes, cells, tissues and organs in living animals. This can only be achieved to a limited degree with organoid cultures and to no real extent in cell cultures.

Reduction

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

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

We keep a database of all animal use which provides us with historical and current usage numbers to base projections on.

Based on our previous experiments, we estimate this is the maximum number of animals we will use over 5 years.

Half of these will be breeding animals which are used to lay eggs for our embryo experiments.

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

We ensure that the minimum numbers of animals are used by preparing our experiments ahead of time and using proper statistical methods, keeping healthy animals, using the fewest animals needed for reliable results, and when possible using fish in the embryonic stages.

In the majority of cases, embryos are used before they reach protected status, which does not contribute to animal use numbers.

We have access to our unit's statisticians and experimental design experts and to seek their collaboration before data collection begins.

Strategic planning of different phases of projects helps to avoid spikes in demand for embryo production by staggering use.

This license also includes a new method for generating genetically modified zebrafish that will reduce the number of animals needed to maintain the genetic mutation.

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

Before we make a new genetic line of zebrafish we make sure it does not already exist somewhere in the world.

We breed only the animals we will need to use or to increase or replace our breeding stocks. The number of adult animals required to generate the embryos can be kept lower by excellent welfare standards which keep the animals at maximum fertility.

If a genetic line of zebrafish is not being used, we will freeze the sperm and keep it until the line is needed again.

Facility staff coordinate with researchers to establish communal animal pools, which are shared by multiple individuals, to prevent individual researchers duplicating animals unnecessarily.

Refinement

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

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

We use zebrafish because they develop cancers that are highly similar to human cancers. Like us, they are vertebrates, and they share many genetic and anatomical features with humans. Zebrafish are not mammals, so they are less similar to us than other animal systems, such as mice. However, we cannot use non-vertebrates (such as worms or flies) because these animals do not develop cancers that have the clinical features of human cancers.

Unlike most other animals used in the laboratory, zebrafish embryos are externally fertilised and are transparent during early development. This allows for imaging of cell and tissue development in a living animal without harming it.

Pain will be controlled by using anaesthesia and analgesia.

We will not keep sick fish or animals with old age related health problems.

We have three dedicated staff looking after the zebrafish to maintain their health. Any fish that is no longer able to swim well, or has evidence of suffering, or additional unexpected signs of disease or illness will be killed in a humane method.

We have a highly skilled staff that is focused on improving their skill set to refine experimental procedures and handling to reduce stress to the fish.

We will stay up to date and well informed on all animal welfare developments relevant to zebrafish and will communicate this information to everyone working with the fish.

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

The majority of experiments performed are on embryos on the first 4 days of development before they reach protected animal status. This is already considered non or very low level of sentience.

The majority of procedures on this licence relate to the fact that the adult fish carry a genetic alteration. The majority of these animals experience no suffering from this genetic alteration that they carry.

For the minority of experiments that may cause suffering to fish, a living vertebrate animal (comparable to humans genetically and in terms of organ development) is essential to study disorders which impact humans through complex interactions within the body. Alternatives to study human genetic disorders in a different vertebrate model would be animals which are arguably more sentient (e.g. mouse) and therefore the alternatives would involve increased potential for suffering.

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

Unlike most other vertebrate model systems used in the laboratory, zebrafish embryos are externally fertilised and are transparent during early development. This allows for detailed visualisation of cell and tissue development in a living vertebrate animal. This also allows for longitudinal (multiple time points from one animal), non-invasive studies (pre free feeding) which in other species would require multiple animals to be culled for each time point (including the mother).

Pain will be controlled during fin-clipping by general anaesthesia and analgesia. Users will be encouraged to use, and offered training in, alternative methods of genetic material collection such as embryo fin-clip and adult surface swab.

Our colony management database allows us to closely monitor the age of all fish and plan breeding accordingly to avoid keeping older fish while waiting on the next generation to mature, thus minimising suffering by avoiding ageing related conditions.

Adverse effects from a genetic mutation will be minimized by maintaining the fish as heterozygous (partial, rather than full mutant) lines if possible, and/or raising fish in areas of reduced daily stress and noise (e.g. darkened tank, reduced water flow, away from general traffic). Daily assessment of behaviour and mortality for each line will make it clear if any lines are suffering adverse effects.

Access to the fish facility will only be given to those who genuinely require access and pass internal training, minimising disturbances.

The facility manager is a nexus for increased coordination within the facility (having a central overview of activities in the zebrafish facility allows for coordination of activities between otherwise unconnected labs) and between other zebrafish facilities, for information exchange and to minimise duplication of lines.

Improvements in zebrafish husbandry are constantly being sought and applied, as well as the continued professional development of staff. The zebrafish staff, involved in service provision, are of a high calibre and their skills are relevant and up-to-date, ensuring minimal number of animals are required to generate new lines. The experience and focus of these dedicated staff on animal welfare is above what could reasonably be expected of individual labs.

New researchers or researchers who are new to zebrafish will have experienced staff to support them to efficiently and ethically make use of zebrafish.

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

The majority of the animals on this licence will not undergo experimental procedures therefore refinement largely relates to the welfare and husbandry the animals experience throughout their lives.

We take guidance from the peer reviewed guidelines of "Zebrafish: Housing and husbandry recommendations" which was published by a group of experts assembled from the 2 leading international bodies concerned with use of zebrafish as animal models in science.

The dedicated team of experienced zebrafish technicians who care for our animals are trained to high standards of animal welfare and ethics as they have received training for personal licences, project licences and to hold the position of Named Animal Care and Welfare Officer. This training is approved by and assessed on behalf of the Home Office.

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

The facility manager and Named Animal Care and Welfare Officer will stay abreast of developments in animal welfare and legislation (such as by attendance of events specifically for fish facility managers and those directly involved in promoting animal welfare). The dedicated zebrafish facility manager will have a greater and constantly updated knowledge of zebrafish husbandry, welfare and disease than could reasonably be expected of group leaders. The same is true for knowledge of legislation. The facility manager is ideally placed to act as liaison to new or established researchers within the facility,disseminate any important information regarding welfare and legislation and guide how it is put it into practice.

The manager of the facility is a member of the 2 leading zebrafish focused international organisations and attends their regular interactive meetings which disseminate the latest advances in welfare and husbandry.

36. Harnessing the lymphoid tissue niche to enhance anti-tumour immunity

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

cancer, immunology, lymphoid tissue, tumour, lymphocytes

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?

Anti-tumour immune responses can eliminate cancer cells in primary or metastatic sites and provide long-lasting protection against relapse. Adaptive immune responses, including anti-tumour immune responses, require the support of lymphoid tissues. We propose to undertake experiments to investigate the lymphoid tissue niche – in lymph nodes and within tumours – aiming to increase numbers of effector T cells, help overcome T cell exhaustion, and support immunological memory.

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

Why is it important to undertake this work?

Close to 1 in 2 people will develop cancer in their lifetimes, and while many cancers diagnosed in early stages can be successfully treated with combinations of surgery and

standard chemotherapies, there are still many cancer types where treatment options are limited and mortality rates are high. Cancer treatment places a significant burden on patients, their families and carers and the side effects of standard chemotherapies can mean significant disruption to peoples work and home lives, so the need for novel treatments is urgent.

During the development of cancerous growths, the body's normal immune responses are suppressed, allowing unregulated abnormal growth. The development of immunotherapies in the form of cytokines, monoclonal antibodies, cancer vaccines and genetically-engineered T cells work to counteract immunosuppression in tumours and has revolutionised cancer treatment. The potential power of immunotherapies is the abilty to target cancer cell in both primary sites and metastatic sites, potentially leading to full remission for patients. Currently immunotherapy approaches do not work for all patients and we have not yet harnessed their full potential.

Current immunotherapies work on the premise that sufficient numbers of effector T cells, with the specificity to recognise tumour antigens, are able to penetrate tumours and maintain function.

However, there are many other approaches we could harness to develop novel types of immune regulatory drugs which require novel research to explore.

Our approach is to understand how the non-immune cells such as fibroblasts in tumours can be reprogrammed to attract and support immune cells function within the tumour tissue. This is a novel approach which we hope will be widely beneficial to a range of cancer types and a large population of patients in the future.

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

This programme of work will lead to better understanding of the protective lymphoid tissue niches supporting immune function; both in lymph nodes and in the tumour microenvironment.

The results of this project will be published in peer-reviewed, open access journals. These data will be used to predict patient responses to immunotherapy and find druggable targets to reprogramme fibroblastic stroma in the tumour microenvironment; to convert immunosuppressive myofibroblasts and inflammatory cancer-associated fibroblasts into immune-regulatory phenotypes which can support tertiary lymphoid tissues.

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

The immediate beneficiaries of these output will be other scientists and academics who will use the knowledge gained to shape their own research projects and progress the field of research.

This programme of work will lead to better understanding of the protective lymphoid tissue niches supporting immune function; both in lymph nodes and in the tumour

microenvironment. In the longer term, harnessing the full potential of immunotherapy will cure more patients and will lead to shorter treatments and less surgery.

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

The work that this project will support will include collaborations with clinical scientists, other academics in life sciences and engineering.

All new knowledge will be published in open access journals and presented at appropriate scientific conferences.

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.

Complex processes such the interactions between a tumour and the immune system cannot be satisfactorily modelled in vitro – therefore it is necessary to use animal models.

The mouse is the only mammalian species for which there exists a large number of transgenic models and techniques are widely available for generation of novel transgenics. The mouse's immune system functions in a similar way to the to human's and therefore they provide a powerful tool for modelling human disease and are therefore the model animal of choice.

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

For tumorigenesis studies, mice will be injected with cancer cell lines and tumour growth and immune infiltration will be assessed. For example, tumours will be imaged at single cell level with enough detail to understand cell-cell interactions and signalling event between cancer cells, stromal cells and immune cells. Tumours will also be digested to generate single cell suspensions for anlysis by flow cytometry, allowing us to quantify the number and cell phenotype of any tumour, stromal or immune cell population in the tumour. We are also able to undertake live imaging studies in surgically exposed tumours or live tumour slice cultures to see and quantify the dynamic movement of immune cells within the tumour. Combinations of these experimental readouts will help us to understand at a cellular level how the tumour growth is regulated by the immune system. We are able to engineer cancer cell lines and stromal cells to express different signalling molecules using genetic tools to test the function of pathways in vivo. Typically these experiments run over 2-4 weeks duration.

It is important that we can combine and compare our tumour studies with understanding of normal immune function. To examine immune responses mice will be immunised with known antigens and a range of adjuvants. These experiments may be done in isolation or combined with tumourigenesis studies above. To alter immune function, or lymphoid tissue stromal function, genetically modified mice will be used. Tissue collected from these experiments will be used for single cell analysis and imaging studies to understand the complex interactions between immune cells, stromal cells and cancer cells.

No mice will be reused in this project.

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

Most of the experiments described in this application require the use of genetically-modified mice that express forms of genes that can be switched on or off at specific times. Therefore these mice are mostly indistinguishable from any other mouse, and undergo no adverse effect as a result of their genetic mutation. Other mice express genetically labelled cells that also have no observable phenotype, but allow us to see them using microscopy since they express a fluorescent signal.

Most animals bred in this programme will be used to produce cells or tissues for additional experiments carried out in vitro. Some of the experiments will involve manipulation of genes in vivo that will result in changes to gene expression in tumours or lymphoid tissue. Other experiments involve injections of tumour cells to assess the ability of the transplanted cells to induce tumours and spread. These animals will be monitored regularly and any animals showing signs of distress will be humanely culled. Signs of distress would include: failure to eat or drink normally, loss of normal mobility, hunched back or lethargy. Based on previous work, the likelihood of distress such as listed here in the animals is low and the maximum severity level is judged as moderate.

Some experiments involve in vivo imaging of cell migration. These animals will be anaesthetised throughout each procedure and will be culled at the end of the imaging experiments, if surgical methods were used to expose the tumour site.

Animals will not be allowed to develop tumours greater than 1200mm3. Since tumours will be injected into sites external and visible to researchers these can be monitored by checking the mice regularly and making measurements with simple tools such as calipers. Within these small tumour size limits mice usually show no changes to behaviour.

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

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

Mice used for breeding and to generate tissue samples we expect mild severity.

Mice used for tumorigenesis studies will be classed as either mild or moderate severity. Any mice bearing a tumour will be classed as moderate severity (>90% of animals on this protocol will develop a tumour). If mice are used as controls (injection of a vehicle solution containing no tumour cells) or fail to develop a tumour they will classed as mild severity (<10% of mice).

For immunisation studies any mice experiencing inflammation and the induction of an immune response following immunisation will be classed as moderate (>90% of animals). If mice are used as controls (injection of a vehicle solution) they would be classed as mild severity (<10% of mice).

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

- Killed

Replacement

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

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

Complex tissue scale processes such a lymph node swelling and full scale systemic immune responses cannot be satisfactorily modelled in vitro, therefore it is necessary to use animal models.

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

We do work in vitro using cell lines to investigate some of the processes involved in cell-cell interactions such as cell shape rearrangements. We also use 3D coculture systems for both immune cell/stromal cell interactions and immune cell/tumour interactions. These model systems can identify important signaling molecules and pathways of potential relevance.

Why were they not suitable?

Many effects measured in vitro are not fully reproduced in vivo due to the additional complexity. Therefore it is always critical to test our models in animals.

Reduction

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

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

The proposed experimental designs and methods of analysis have been discussed with our experts in statistics. Factorial experimental design will be used wherever appropriate to maximize the information obtained from the minimal numbers of animals required.

From experience with the experimental systems we plan to use, we predict that 6-8 animals will generally be required per treatment group to obtain statistically relevant datasets. In cases of tumour development, this may need to be slightly higher since not all animals may develop tumours. The in vivo experiments outlined in this project make up only one part of our research programme.

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

As the project licence holder, I will ensure that personal licence holders working on this project are appropriately trained to ensure a high success rate and to minimize the number of experiments that require repeating.

For example, PIL holders will be encouraged to use the EDA (NC3Rs) to calculate mimimum treatment group sizes based on effect sizes, variability and statistcal power.

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

To ensure efficient breeding, we use both male and female mice in all experiments.

Where we breed genetically modified animals with heterozygous transgenes, the mice negative for the transgene are used either as littermate controls for the expeirment or used in other parallel studies where the transgene is not required in the experimental design.

To optimise and maximise the data collected from each animal we make sure to use all tissues for multiple analyses. For example, tumours dissected will be weighed, cut into portions and used for both imaging studies and flow cytometry analysis - which allows us to match cell numbers, phenotypes and spatial arrangment 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.

This project does not include protocols where the outcome is expected to be severe. However the biological resources services oversee a comprehensive health monitoring programme, and therefore where adverse effects may develop, they will be recorded, and appropriate action will be taken to minimize severity or to end the protocol.

To minimise pain, suffering and distress we have implemented tunnel handling for all proceedures. Injection of tumour cells is carried out under anaesthesia. Further, intraperitoneal injections have been replaced with gavage dosing which minimises the chance of injury occuring.

All procedures will be performed by competent staff and we adhere to a carefully monitored training process before any PIL holder is signed off as competant for any proceedure.

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

The mouse immune system is similar to humans and therefore they provide a powerful tool for modelling human disease and are therefore the model animal of choice, before clinical trials can be undertaken.

The mouse is the only mammalian species for which there exists a large number of transgenic models available and techniques are widely available for generation of novel transgenics.

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

Before starting any study, a pre-study form is filled out, checked and signed off by both the PPL holder and the staff for the animal facility. This ensures high standards are maintained, and identifies opportunities for further refinement.

To minimise welfare costs animals involved in protocols of this project are regularly monitored for adverse effects by the PIL holder and the staff of the animal facilities. 2 additional PIL holders are always listed for each study to ensure animals will always have a dedicated PIL holder to respond to any issues arising.

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

We work to the AWERB guidelines as published.

We also refer to Workman et al. guidelines on the welfare and use of animals in cancer research. https://doi.org/10.1038/sj.bjc.6605642

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

Advances in the 3Rs are shared widely through the institution via email communications, updates to policies and through invitations to webinars where appropriate to ensure all PPL and PIL holders adhere to the highest and most up to date standards.

37. Impacts of increased overwinter temperatures on the behaviour and physiology of a temperate reptile

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Reptile ecology, Climate change, Winter warming, Animal Behaviour, Animal Physiology

Animal types	Life stages
Podarcis muralis (Common wall lizard)	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 effects of increased overwinter temperatures on common wall lizard physiology and behaviour.

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

Why is it important to undertake this work?

Environmental change is causing temperatures to rise across the globe - how this will affect the distribution and abundance of species is therefore an important current focus for ecologists worldwide. An under-recognised aspect of climate change is increased overwinter temperatures. While research has focused largely on the impacts of hot periods (i.e. summers) getting hotter, winter temperatures have in fact been rising at a faster rate than summer temperatures over the last century.

Ectothermic species such as reptiles are especially vulnerable to projected thermal shifts given their physiological reliance on ambient temperatures. However, reptile responses to climate shifts have received little attention, and existing work has been heavily biased towards the summer active season. Reptiles at temperate latitudes often rely on an overwintering period characterised by temperature- sensitive behavioural and physiological torpor (including reduced activity, and cessation of feeding).

Evidence suggests that milder winters could disrupt overwintering behaviour, with lasting carryover effects on phenology, breeding success, and tolerance to stressors. Nevertheless, due to the scarcity of data, how reptiles in temperate regions such as the United Kingdom will respond to rising winter temperatures remains unknown. This makes it difficult to predict impacts, project future population trends, or understand habitat requirements that could be taken into account in conservation management.

This project aims to address this major gap in knowledge. Using an invasive lizard in the UK, the common wall lizard (Podarcis muralis), we will investigate how increased overwinter temperatures affect their physiology (body condition, stress levels, reproductive output) and behaviour. The common wall lizard is an ideal species for this work. In the UK it is at the northern edge of its range and relies on a period of overwinter torpor for survival, meaning that changes in winter temperatures are highly physiologically relevant for this species. The common wall lizard is not threatened in the UK, and so is a preferred candidate species for this research than native lizards which are of greater conservation concern. However, the wall lizard is threatened in some parts of its European range (e.g. Germany), so our findings will also be of relevance to conservation of the species in parts of Europe where winter temperatures are increasing. Additionally, the common wall lizard is an invasive species in the UK, so understanding the thermal ecology will allow us to also better understand a key factor in predicting its future spread and success in Britain.

This project will represent a major advance in climate change/animal research generally as the effects of winter warming have been little studied in vertebrates overall. This work will also provide an important insight into the likely impacts on British herpetofauna and other temperate European species more specifically.

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

1. New information

This project aims to investigate questions that will result in highly novel data/information at several levels. First, the response of wall lizards (P. muralis) to winter warming has to our knowledge not been tested at all previously despite the potential value in doing so given its ubiquity across Europe. This project will therefore provide valuable and heretofore absent information about this specific species, which could be beneficial in understanding its spread as an invasive species in the UK. Second, the impacts of climate warming on European species has also received relatively little attention (i.e. relative to tropical species), although changes in wintering conditions are likely to be strongest at higher latitudes and therefore particularly risky for temperate reptiles. The data we generate will

therefore contribute important information towards the study of climate warming effects on herpetofauna in previously understudied regions.

1. Publications

We anticipate that this project will result in a minimum of 4-5 publications in scientific journals such as Animal Ecology, Molecular Ecology, Conservation Biology, or Biological Invasions. We will additionally write a report of our findings for the Amphibian & Reptile Conservation Trust (ARC) for dissemination on their website and within their broader non-academic network.

2. Other communications

Results will also be communicated to national and international scientific audiences at relevant congresses such as the World Congress of Herpetology, and the British Ecological Society. The PhD student collaborating with this project will additionally participate in outreach events organised by their funder. The first outreach events for children have already taken place in July 2023, and will be repeated throughout the project, providing the opportunity to explain the research project and key results to children to raise awareness around invasive species, global climate change, and its effects on species.

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

Summary of outputs:

Short-term (1-2 years): preliminary congress communications and publications, feedback and reporting to non-academic partners.

Medium-long term (3-5 years): high-impact publications, international congress presentations, feedback and reporting to non-academic partners, proof of concept or template for others studying the impact of winter warming on herpetofauna.

Key beneficiaries:

- Scientific community: Our results will benefit conservation and animal behaviour scientists and comparative physiologists in: i) increasing our understanding of the response of a temperate reptile species (e.g. P. muralis) to warming winter regimes, in terms of both physiological and behavioural responses, ii) understanding the thermal traits underpinning the success of a non- native species, and iii) providing information that will be used in future predictions and modelling of biodiversity responses to global climate change, in particular vertebrate ectotherms. Our work will also serve as a proof of concept or template for other research groups seeking to study the impact of winter warming on reptiles in temperate regions.

- Non-academic/Wildlife Charity partners: Our results will benefit non-academic partner institutions such as wildlife charities, including but not limited to our collaborating organisation the Amphibian & Reptile Conservation Trust, in two ways. First, we expect that our results will be useful in helping to understand the risks facing

all reptile species in the UK as a result of climate warming. Although we are only focusing on one species, the physiology and ecology of wall lizards is similar enough to other lizard species in Britain that it will be possible to broadly extrapolate our findings to indicate either that winter warming is or is not an immediate threat to reptilian biodiversity. Second, our results will provide valuable insight for ARC on the status of the common wall lizard as an invasive species in the UK, specifically, its thermal preferences and response to different overwintering temperature regimes. This will be helpful in informing their future work, for example, distribution mapping and predictive modelling of phenology and distribution of this species, in addition to possible mitigation measures where they may be outcompeting native, threatened species.

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

The outputs of this work will be maximised by: 1) publication of all results in scientific journals to ensure wide dissemination of knowledge in the scientific community; 2) collaboration with academic and non-academic partner institutions, most particularly the Amphibian & Reptile Conservation Trust, which will facilitate data- and information-sharing; 3) dissemination of results at national and international congresses; 4) dissemination of information and results via popular media where appropriate; 5) inclusion of results in teaching material; 6) collaboration with PhD funding body to disseminate the results to a general audience, in particular to children.

Species and numbers of animals expected to be used

- Reptiles: No answer provided

Predicted harms

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

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

The common wall lizard has been chosen for this study because they are the most relevant species in the context of the research question (a lizard species found in the UK) that is not threatened (as UK native species, such as the common lizard and the sand lizard, are). Additionally, the common wall lizard has a large geographical distribution (mainly Europe) and high cold tolerance and broad thermal breadth, making them an ideal candidate for our research aims. Alternatives such as laboratory- or captive bred lizard species are not suitable given our focus on natural overwinter conditions likely to be experienced by animals in the UK. Wall lizards have been successfully used in captive experimental studies across Europe, and the same protocols (in terms of sampling) that we intend to use have also been used on this species successfully in the UK before.

We are using only adult individuals for procedural work. Again, this is because this is the most relevant stage in the context of our research question, as most individuals experience overwintering as adults, and because we are interested in the consequences

of winter condition on reproductive traits (so animals must be of reproductive age). Only using adults also reduces variation in our study animals, which allows us to reduce the total number used. Juvenile animals are also too small in size for collection of a suitable volume of blood for hormone and antioxidant analysis.

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

The establishment maintains appropriate housing settings and has experienced staff with a strong background in lizard husbandry and care conditions. In brief, the wall lizards are housed in transparent plastic boxes (approximate dimensions 55Lx35Wx40H cm) during the active season. Each box contains a thin layer of sand and a water dish, and stacked bricks/plastic tubes, rocks and a quartered log inside the box to provide shelters and platforms for basking. Heat and natural photoperiod are provided by an 80w UV basking lamp hung above each terrarium and the main room light. A maximum of 4 individuals are housed in each box. There is no known mortality rate for animals in captivity, but pilot data suggests that a loss of less than 5% is expected during the overwintering period, given that we can only estimate animal age at capture (the expected lifespan of a wall lizard is 4-5 years).

Capture

Wall lizards will be captured using the most common method of lizard capture, noosing or "lassoing", whereby a small loop of dental floss or fishing line attached to the end of a fishing rod/pole is passed over the animal's head during their natural basking behaviour (when the head is raised from the substrate on which they are sitting) and gently tightened by pulling towards the researcher. This is sufficient to lift small-bodied lizards off their perch or basking spot without harming them; they can then be safely dropped into a bucket (the loop will release them as soon as tension is removed), and then be placed in individual cloth bags/plastic boxes for transport to the establishment. During transport, bags/boxes are stored in a dark cooler which calms the animals, and they are misted regularly with cool water to provide moisture.

Overwintering

The overwintering practices we use follow a published protocol from a collaborating research group in Porto (Portugal; Barrosso 2023). Lizards will be overwintered under three experimental temperature regimes (ie. "cool", "warm", "fluctuating"). Animals will be randomly allocated to each of these three treatment groups while ensuring an equal distribution of male and female individuals within each. While these will differ (by a max of 4-6 ˚C), temperature ranges will all fall within normal wintertime temperature ranges in the UK. In the home range of the population from which the animals will come, the temperature range between December and March is 1.58 - 11.40˚C (Bournemouth airport weather station, metoffice.gov.uk).

Prior to entering the overwintering period, lizards will be acclimated over a period of 2-3 weeks to a gradually reduced room temperature and light availability, which will trigger

natural overwintering behaviour (such as food refusal and reduced activity). Experimental overwinter periods will be seasonally appropriate (i.e. will fall between November and April) and will last for approximately 4 months, as is standard in regular lizard husbandry.

During this period, lizards will be housed in plastic tubs within ventilated refrigerator units, and will have access to shelter and water. All other variables (photoperiod, food and water availability) will be the same across units and treatments such that only temperature varies between treatments. Housing will be as naturalistic as possible, for example including sphagnum moss which provides moisture, and naturalistic substrates for shelter and basking. Lizards will be disturbed as little as possible over the overwinter period, but will be visually checked daily, and weighed to check condition (once monthly so as to avoid frequent handling, with the expectation that some mass loss is expected due to normal food refusal during this time). Additionally, infrared surveillance cameras will be used to constantly monitor and record at least one tub per unit to allow for a more thorough assessment of lizard behaviour; these video records will be assessed regularly for signs of behavioural dysfunction (e.g. signs that torpor has not been achieved), or distress.

Lizards will be removed from overwintering conditions in Spring, again with a 2-3 week period of re- acclimation to gradually increased temperatures and light, during which food will also be offered (reptiles naturally do not eat during the overwintering period).

Blood sampling

To measure the physiological effects of winter warming on wall lizards, we will collect small volume blood samples from each individual. The procedure will only be performed once per year during the duration of the project (5 years) – so 5 blood samples per individual in total with a full year separating each sampling session. The final blood sample will be taken post-mortem, thus reducing the total number of procedures per animal to 4. Blood samples will be used to assess levels of glucocorticoid hormones, and to assay for markers of oxidative stress, with both representing measures of the physiological and energetic cost of overwintering at warmer temperatures.

Tissue sampling

Due to the volume of blood required for hormone assays, we will also collect small tissue samples to allow genomic sequencing of individuals which will allow us to test links between the behavioural data collected and genomic traits/population ancestry (it is not possible to collect sufficient blood to allow all physiological/genetic tests to be carried out on the same blood sample). Tissue will be taken from the tail tip: this can be done quickly and easily without the need for anaesthesia (which would likely cause more harm/distress) by gently squeezing the tip of the tail (~1cm from the tip) with a pair of tweezers, utilising the lizard's natural autotomy response (the tail tip will drop off at this point, with no/minimal blood loss). Tail sampling will only be performed once during the entire duration of the project (5 years) and only on individuals which have not previously lost tail tissue in the wild (which can be visibly determined).

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

We expect no adverse effects of overwintering treatment, as this is part of the animals' natural life history cycle and our treatments will recreate temperature ranges that they would experience in their home ranges in the wild. Some mass loss is expected due to natural food refusal induced by low wintertime temperatures.

Adverse effects of blood and tissue sampling could include brief pain/distress during the procedure itself, and a short period in which abnormal behaviour (e.g. increased use of shelters, "hiding") may be observed. These adverse effects will be extremely transient: any associated pain/distress will last for seconds to minutes, and abnormal behaviour 1-2 days at most, if at all. Overall, we expect that these procedures will have no adverse effects that are more than mild and transient.

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

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

Blood sampling: 100% mild

Tissue sampling: 100% mild

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

- Killed

Replacement

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

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

Our research question specifically pertains to the experience of animals under natural conditions, i.e. conditions during winter/the overwintering period. This requires the use of animals, and more particularly a species that naturally would experience overwintering as part of its natural seasonal cycle. It is our aim that the results of our study (i.e. temperature effects on behaviour/physiology) could be used in the future to parameterize theoretical models that could help to estimate the same effects in other species or under different temperature regimes, however, this level of mathematical modelling (or more importantly, the data required to power it) does not yet exist.

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

This project is not possible without the use of animals for the reasons stated above.

Why were they not suitable?

It is not possible to answer this question using non-animal models, as the relevance of overwintering can only be experienced in intact, living animals.

Reduction

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

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

First, we reviewed published studies of experimental tests on reptile physiology, including those testing the effects of climate change and winter warming specifically. Published studies testing two alternative treatments in a homogenous population have had sample sizes of around 15-20 individuals per treatment. We propose three treatments (cold, warm, and fluctuating winter temperatures that differ by a max of 4-6˚C) in order to test a more nuanced question about temperature variation during the overwintering period (whether differences are due to constantly warmer temperatures, or to a more naturalistic fluctuation from cool to warm, compared to a cool overwintering period). Given this additional complexity we intend to use 20-25 individuals per treatment. This also allows us to better control for variation between male and female individuals. Although using only one sex might have allowed us to slightly reduce these numbers, we have opted to use both sexes as keeping lizards in single sex groups for prolonged periods moves significantly away from the natural behaviour of this species, where males and females encounter each other frequently on shared territories and basking spots, and therefore could be likely to influence our results.

Second, I used my own experience of the level of variability that is usual in assays of the sort of physiological markers we intend to use, specifically in glucocorticoid hormone assays, which I have used in multiple studies. Given typical variation from my previous experimental work, we calculated the minimum number of animals to use whilst ensuring that the results are statistically robust.

Capture success is likely to be high given the density of animals at our proposed field site.

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

As well as using what we estimate as the lowest possible number of animals to still ensure robust and publishable results from our data, we have taken steps to reduce the number of animals used through careful optimisation of our experimental design. We will use animals taken from a single source population in order to reduce extra variation that might have

required additional animals to account for statistically. We have opted to use only low-severity procedures. Blood and tissue sampling procedures are mild so both sample types can be taken from each animal without causing additional suffering to separate animals for each sample type.

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 optimised our protocols in order to provide as much use for samples collected as possible. For example, blood samples can be used for hormone and oxidative stress assays, and tissue can be used for additional oxidative stress assays as well as the primary purpose of genetic sequencing.

Sequences generated from this tissue will be used to answer questions directly related to the main objectives of this project, but can also be used in the future for other purposes (for example, assessing genetic relatedness within lizard populations).

Refinement

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

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

The animal model we will use is the common wall lizard (Podarcis muralis), a widespread species listed as "Least Concern" in the IUCN Red List. Able to colonize a wide array of habitats and adapt to a wide range of conditions, the common wall lizard is an excellent model for climate change studies, and in the UK, a suitable alternative to using native species, which are of greater conservation concern.

They display overwintering behaviour (behavioural and physiological torpor) when environmental conditions are harsh and food resources are absent/scarce. During the overwintering period, their activity is significantly reduced and they rarely, if ever, feed. They also adapt readily to captive conditions and are used in laboratory studies frequently across Europe.

Overwintering captive temperate reptiles such as the common wall lizard is standard practice in reptile husbandry. We have refined basic overwintering methodology by including a long acclimation period of up to three weeks during which photoperiod and temperature will be gradually reduced. This will promote a naturalistic induction of physiological and behavioural torpor in the lizards as well as giving us a suitable length of

time to identify if torpor is not being achieved (ie. if an animal remains highly active and seeking to eat), in which case that animal can be removed from the experiment before any suffering occurs due to further temperature reduction.

Methods for blood and tissue sampling have been carefully selected and refined to cause the least pain and lasting harm to the animals. This is evidenced by their widespread use for these purposes in a large number of studies (blood sampling e.g. Sacchi et al 2018 Ecological Indicators 93: 856-863; Storniolo et al 2022 Parasitology 149:1179-1185; tissue sampling e.g. Oskyrko et al 2022 Biodiversity Data Journal, 10, p.e90337; García-Muñoz et al 2011 Acta Herpetologica, 6(2), 223–227; Michaelides S, While G, Bell C, Uller T 2013 Biological Invasions, 15, 1101–1112; Michaelides, S.N., While, G.M., Zajac, N. and Uller, T., 2015. Molecular Ecology, 24(11), pp.2702-2714). Specifically, collection of blood from the postorbital sinus (accessed via the mouth using a capillary tube) is preferable to withdrawal of blood intravenously for a number of reasons. First, it does not require lengthy restraint as the use of a needle would. The lizard can be handled briefly and gently in a natural posture (without the need to be held on its back). Finding the tail vein is also difficult in relatively small-bodied lizards like P. muralis, while the postorbital sinus is very easy to access and yields a suitable amount of blood much more quickly, lessening the total amount of time required for handling.

Collection of tissue from the tail tip using natural autotomy is also preferable to other possible methods, most of which are terminal. Forced autotomy reduces blood loss and the potential for infection which is higher relative to when using surgical instruments to remove tail tips. This has been found to be a low- stress procedure (García-Muñoz et al 2011 Acta Herpetologica, 6(2), 223–227) and has been frequently used as a sampling method in lizards in the scientific literature and is generally considered the most reliable technique to gain DNA-templates for genetic analyses in reptiles (Schulte et al 2011 North-Western Journal of Zoology, 7(2), 325-328).

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

First, our research objectives require us to use animals that would naturally experience overwintering and enter a torpor state, requiring them to be a) adults, and b) of this level of sentience (ie has to be an overwintering species for this study to be ecologically relevant).

Second, a key part of this project is linking physiological changes due to variation in overwintering temperature with behavioural changes, such as activity levels during the overwinter period, and thermoregulatory preferences and activity in the post-overwintering period. It is therefore not possible to use 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 animals are rigorously monitored as part of their regular husbandry at the establishment (i.e. checked daily by trained technicians with substantial reptile experience, and remotely using camera equipment where appropriate). To refine our protocols, following the

procedure, the animals will be closely monitored to allow intervention if they show signs of distress or cage mates show aggression, and housed individually on a temporary basis if appropriate. This will ensure that severity levels are remaining at mild as forecast.

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

We will follow established guidelines for ensuring maximum refinement of our protocols at every step, such as NORECOPA's PREPARE guidelines and the Humane Endpoints website of the 3Rs Centre at

Utrecht University. For reptile-specific guidelines, we will also follow the Guidelines for the Use of Love Amphibians and Reptiles in Field and Laboratory Research, published and regularly updated by the Herpetological Animal Care and Use Committee of the American Society of Ichthyologists and Herpetologists.

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

I have already subscribed to the NC3Rs e-newsletter to keep up to date with new publications relating to refinement in experimental procedures and methods for reducing animals used in experiments, as well as continuing discussions about improving research practices with colleagues, collaborators, technicians working at my establishment and in the broader field, and the Named Animal Care and Welfare Officer (NACWO) and Named Veterinary Surgeon (NVS).

38. Investigating pericytes in chronic lung 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

asthma, inflammation, pericytes, stem cells, fibrosis

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 overall objective of our investigations is to identify the mechanisms that trigger tissue fibrosis, i.e. scar formation. We will achieve this by interrogating the function of pericytes, a type of tissue-resident progenitor cell. We will focus primarily on the regulation of pericyte migration and interactions with other cells at sites of 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?

These studies are important to identify and characterise the key molecules responsible for the acquisition of a scar-forming characteristics in pericytes in response to chronic

inflammation. This will enable us to understand the functional roles of key molecules involved in inflammation that impact on the function of pericytes, and to target these mechanisms for the development of new medicines for the treatment of tissue scarring. Additionally, these studies will provide a greater understanding of the mechanisms regulating scar formation by pericytes in the context of chronic inflammation.

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

My research group and other have conclusively demonstrated that pericytes are major contributors to the pool of scar-forming myofibroblasts in fibrotic tissue. However, the mechanisms by which pericytes transform into myofibroblasts and migrate into inflamed tissue are poorly understood. This project will provide a deeper understanding of the mechanisms involved in inflammation-driven fibrosis, which is fundamental to analysing the causes of fibrosis on the one hand and manipulating cell behaviour on the other. This understanding is critical for the design of novel therapeutic strategies for common inflammatory diseases involving tissue fibrosis (e.g. liver cirrhosis, emphysema, cardiac fibrosis, kidney fibrosis), for which there is no effective treatment. Outputs of this project will include peer-reviewed, open access publications, conference presentations, and novel drug discovery efforts.

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

In the short term, beneficiaries will include the scientific community interested in chronic diseases involving tissue fibrosis, as the results of this project will provide critical mechanistic insight into this process. Long-term beneficiaries include patients with fibrotic disease and their carers/families, the medical community, and the pharmaceutical industry as a result of novel drug discovery efforts arising from this project.

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

Collaborations have been established to extend the findings from this project focused on allergic asthma to other chronic fibrotic diseases, i.e. chronic obstructive pulmonary disease Insights from this project will be used to inform our approach on these other research programmes.

Species and numbers of animals expected to be used

- Mice: 600

Predicted harms

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

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

We have chosen mice as this species reflects many important features of inflammatory lung disease in humans. We have also chosen an allergen exposure model that, although it exhibits the salient features of human disease in treated animals, is the least severe and does not impose distress on the mice. Typically, young adult mice will be used in these studies (with a balanced gender ratio, initiated into the protocol at 6-8 weeks of age) to best reflect the disease characteristics seen in adult asthma patients

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

The ‘typical experience’ of a mouse subjected to our chronic allergen exposure protocol is similar to those of a human with mild-to-moderate asthma. The model consists of either five weeks (to induce early stage disease) or seven weeks (late stage disease) of allergen extract delivered intranasally under light anaesthesia, five days a week, followed by a two-day rest period. The allergen extract is dropped directly onto the nostril while the mouth is held closed to ensure aspiration. Initially, responses to allergen exposure include sneezing and nose rubbing (after ~10 exposures), followed by the development of laboured breathing with a hunched posture (after ~20 exposures). In some animals at the end of the late stage disease protocol (~35 exposures), wheezing may be audible. These five respiratory symptoms generally subside within 5 minutes of exposure. At all other times except for the period immediately following allergen exposure, animals demonstrate normal activity and behaviour.

The disease severity/distress of animals subjected to allergen exposure is monitored following allergen delivery using a scoring sheet that considers factors such as appearance, food/water intake, clinical signs, behaviour/activity, as well as the five respiratory signs described above.

In addition to the allergen exposure protocol, mice may be subjected to additional procedures, i.e. blood sampling, non-invasive plethysmography, and substance administration. None of these procedures are expected to induce more than transient discomfort and no lasting harm is anticipated.

At the end of the experimental protocol, mice will be killed using a Schedule-1 method.

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

Substances. The substances to be used include agents that interfere with inflammatory pathways. Doses will be no more than the maximum tolerated dose (MTD – defined as that associated with weight loss of no more than 10%). There may also be mild discomfort at the site of administration. In the event of major inflammation, ulceration or swelling sufficient to cause distress at the site of administration, animals will be humanely killed. In our experience, substances which affect circulating cell numbers should not immunocompromise animals, but barriered housing will be provided if deemed necessary.

Injection. Animals undergoing injection with substances may suffer mild and self-limiting fever. There is a small risk of infection associated with the administration of agents intraperitoneally. Animals will be inspected regularly for the above-mentioned signs of ill health or ascites formation in which case they will be humanely killed.

Allergen/antigen administration. Anaphylactic responses during administration of allergen/antigen will be monitored. If necessary, such symptoms can be minimised by the administration of an anti- histamine (e.g. pyrilamine) intraperitoneally. If any animals exhibit respiratory distress, they will be humanely killed,

Withdrawal of blood. Small blood samples will be taken from superficial blood vessels. No adverse effects are expected from this.

General anaesthesia with recovery. There will appropriate maintenance of general anaesthesia using inhaled agents (i.e. isoflurane) and continual monitoring of the animal to ensure complete recovery.

Animals will be returned to the cage during recovery and observed until fully ambulatory. Animals will be humanely killed if they fail to recovery from anaesthesia within a time frame suitable for the anaesthetic used.

Terminal general anaesthesia. In the terminal phase of the procedure, the animal will be insentient throughout.

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

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

Control mice, i.e. mice exposed to sterile PBS (50% of animals in each experiment), are not expected to exhibit any adverse effects.

Allergen exposed mice (50% of animals in each experiment) are expected to experience adverse effects.

It is expected that, in every experiment, 25% of the animals will experience mild severity and 25% of the animals will experience moderate severity effects.

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

- Killed

Replacement

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

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

Due to the multi-cellular interactions involved in the inflammatory response and in inflammatory diseases such as asthma, responses cannot be adequately or fully mimicked by in vitro studies alone. The use of animals enables us to assess molecular interactions resulting in changes in physiology and pathology in the same animal and moreover allow us to study the complex interactions between cells and organs that result in the generation of fibrosis as a consequence of chronic inflammation. The animal chosen for this model reflects many important features of lung disease in humans.

Where possible, we will complement the in vivo work with experiments using in vitro culture systems and we will also take advantage of isolated human cells and cell lines to investigate selected pathways identified in the in vivo models. For example, we will use commercially available human pericytes to optimise all assays before initiating similar in vitro studies on pericytes obtained following allergen challenge of sensitised animals.

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

Some more focused experiments have been moved to an in vitro, three-dimensional organoid cell culture system. Work on our previous license revealed the critical roles of oxidative stress in disrupting pericyte/endothelial cell interactions and the inflammatory pathways regulating pericyte migration.

Detailed mechanistic studies are currently ongoing using our organoid system to query these mechanisms and replace the need for live animals to answer these scientific questions.

Why were they not suitable?

In order to pharmacologically target the immunological mechanisms underlying disease inception, progression and resolution, it is necessary to use whole animals. The complex interactions between cells within different organ systems cannot be replicated using in vitro tissue culture systems. Our experiments rely on the study of integrated organ systems in order to examine the functional roles that key cells and molecules play in immunological events that impact on the downstream pathophysiological features of disease that manifest as disease symptoms.

Reduction

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

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

Using a group size of five with four treatment groups in each experiment (PBS control/allergen exposure + vehicle treatment/intervention) results in a total of 20 mice per experiment. It is estimated that six experiments will take place every year, with a duration of 5-7 weeks each as well as a period for data analysis and reflection. Over the license period of 5 years, this will require 600 mice.

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

In contrast to our previous license, where invasive in vivo methods were used to assess lung function in anesthetised mice, we have reduced the number of mice needed for these experiments by instead adopting the method of precision-cut lung slices as an ex vivo assessment of airway constriction. This method allows us the ability to assess multiple pharmacological mediators of airway contractility using tissue derived from a smaller number of mice (5 mice per treatment group using lung slices vs. 10-12 mice per treatment group for in vivo assessments), representing a 50% decrease in the numbers of mice needed for lung function assessments.

The NC3R's Experimental Design Assistant (EDA) will be implemented to develop a visual representation of planned experiments, and to receive feedback on these experimental plans. This service provides also support regarding randomisation, blinding, and sample size calculations and allows plans to be shared amongst colleagues to enhance transparency and foster collaboration.

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

Efficient breeding with prompt genotyping will be employed to minimise the number of animals used in Protocol 1. Molecular modelling will be used to define a small number of candidate molecules to be tested in vitro and in vivo. In vitro studies will be designed with the support of Reporting In Vitro Experiments Responsibly – the RIVER Recommendations (https://osf.io/preprints/metaarxiv/x6aut/).

Small in vivo pilot studies will be used to assess the validity of these pharmacological targets before progressing to larger studies. Archived tissue may be shared with established collaborators.

Refinement

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

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

We have developed effective and clinically relevant experimental protocols so that we can collect the maximum amount of information (physiological, pathological and immunological parameters) from the same animals in order to minimise numbers. We work closely with animal care staff and veterinary surgeons to ensure the best possible husbandry and welfare for mice under the procedure.

We have chosen mice as this species reflects many important features of inflammatory lung disease in humans. We have also chosen an allergen exposure model that, although it exhibits the salient features of human disease in treated animals, is the least severe and does not impose distress on the mice. For example, in comparison to ovalbumin-driven models of allergic airway disease, mice subjected to our house dust mite extract (HDM)-driven model (5 weeks of daily, low-dose allergen challenges) do not require an intraperitoneal injection containing the antigen and an aluminium salt adjuvant to initiate the disease. This is an important refinement to models of allergic airway disease because the HDM model, which employs exclusively respiratory allergen exposure under anaesthesia, not only more accurately reflects the route of allergen exposure in humans, but importantly also reduces the suffering experienced by the experimental animals by omitting the need for injections.

Additionally, the HDM exposure model induces a less severe form of disease than fungal allergen models (e.g. Aspergillus); this can be considered as an additional refinement to these proposed experiments as the disease model chosen accurately reflects human disease without inducing severe distress and discomfort in animals.

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

The planned experiments require prolonged allergen exposure in adult, immunologically competent animals with a reasonable degree of homology with humans.

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

We have developed a detailed scoring sheet that is used to assess body condition and behaviour in mice, which contains a detailed assessment of respiratory symptoms; this scoring sheet is used to assess the health of animals immediately following allergen delivery until the mice are ambulatory and breathing normally. Our condition scoring system is constantly under review. Additionally, we continue to develop the techniques employing precision-cut lung slices to further optimize the use of animals in this study.

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

The revised ARRIVE guidelines will be followed to ensure quality control in all experiments. A detailed monitoring sheet will be completed for all animals following all interventions to assess the severity level of procedures.

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

The NC3Rs website will be consulted regularly, particularly the section on “3Rs advice for project licence applicants”. Additionally, the project license holder sits on her institutional Animal Welfare and Ethical Review Body (AWERB) and as such is continually apprised regarding 3Rs advances

39. New treatments for postoperative pain

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

postoperative, pain, non-opioid, novel, treatment

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 identify new therapies for the treatment of postoperative pain, which may require validation of the drug target.

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

Why is it important to undertake this work?

Surgery and anesthesia are critical healthcare services that reduce the risk of disability and death for millions of patients worldwide every year. However, postoperative pain is not adequately managed in a significant proportion of patients. Furthermore, opioids are the mainstay of postoperative pain treatment in spite of strong evidence of their drawbacks including addiction liability. Indeed, up to 10% of opioid-naïve patients have persistent opioid use after many types of surgeries. As well as the possibility of addiction, common side effects of opioids include constipation, sedation, dizziness, nausea, vomiting, constipation, and respiratory depression, which limit their usefulness. NSAIDs (eg ibuprofen) and paracetamol also play an important role in postoperative pain control, but are considered useful only in mild and moderate pain, and are frequently used in combination with opioids. Poor control of postoperative pain has many possible impacts including the development of chronic postoperative pain, impaired recovery from surgery and prolonged opioid use. There is therefore a need for new effective, non-opioid treatments. New non-opioid treatments for postoperative pain are being developed, which require testing in translatable animal models before they can be progressed into human clinical studies.

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

The major output of this work is the identification of novel drugs for the effective treatment of postoperative pain. This project licence will generate data that will establish and progress drug discovery projects aiming to produce treatments which are superior, in terms of effectiveness and side- effect liability, to current treatments.

By applying our extensive expertise, data will be generated that will assist our clients in selecting drug candidates with appropriate characteristics, such as increased efficacy and reduced side effect liability in the treatment of postoperative pain (particularly vs. opioid treatment) at an appropriate stage in the discovery process. This will allow appropriate compounds to be selected by our clients and progressed into clinical development.

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

The output from this project contributes to the progression of early stage drug discovery projects by our collaborating organisations. If also successfully progressed through toxicology and clinical assessment, compounds that are active in the models covered by this licence may be used in the clinical treatment of postoperative pain, at which point patients undergoing surgery will benefit. The process subsequent to a positive result in the models covered by this licence takes a number of years.

Our collaborators will benefit by being able to focus their resources on the progression of appropriate agents for the treatment of postoperative pain.

Short term:

- By producing high quality data based on high quality methodology and science, clients will be able to make informed decisions about their compounds.

- The data we produce will enable chemists to produce structure-activity relationships and hence create more drug-like molecules.

- Compounds that are not worthy of progression can be identified and eliminated early in the drug discovery process, resulting in the use of fewer animals overall.

- Worthy compounds can be identified early and be focussed upon, to help speed up their progression. Medium term:

- Using additional measurements of biomarkers, which are often taken during our studies, our clients will receive more information about their compounds prior to development. This will further enhance the generation of structure-activity relationships, as well as provide translational data that can be used for dose ranging in subsequent toxicology and clinical studies, should a compound be progressed into development.

- By having an integrated approach we are able to carry out pharmacokinetic studies (covered by another project licence) that come earlier in a typical discovery cascade. This makes the transition within the drug discovery cascade seamless. Our understanding of the compounds from pharmacokinetic studies is fed into the protocol designs for animal disease model studies. The data generated will therefore be from a consistent approach and will, as a result, be more comparable across studies and hence more valuable to the client.

- The progression of more drug-like compounds into development (increasing the chances of long term success)

Long term:

- Produce data to support the further development of drugs that can benefit patients undergoing surgery.

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

By offering the models as a service we will be collaborating with organisations actively progressing their projects for the treatment of postoperative pain. We will generate reports of the studies for our collaborators, which will be of a standard that is appropriate for the subsequent drug development process and regulatory scrutiny.

Species and numbers of animals expected to be used

- Mice: 800
- 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.

Experiments in this project typically follow test tube (in vitro) studies. However, in vitro methods cannot predict and replace whole animal (in vivo) models, as the technology does not exist to simulate the complexity of the whole body system. New, potential medicines that do merit testing are assessed in rodent models of postoperative pain that detect clinically effective medicines. Adult mice and rats will be used in preference to other species because they share similar pain sensory physiology to humans, and there is a wealth of prior knowledge from data generated in mice and rats that can be used to contextualize results generated by novel entities.

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

The in vivo mice and rat experimental protocols covered by this project model pain sensitivity in naive animals and in animals that have undergone an incision.

In the case of naive animals, after a novel treatment, or vehicle is administered, mouse or rat tails or paws are exposed to an escapable heat source, which is set at a level that won't burn them. The amount of time it then takes the animal to withdraw its tail or paw from the heat source is recorded.

In the case of pain sensitivity following an incision, an animal is habituated on two occasions prior to the incision, to the process use to measure the amount of time it takes to withdraw its paw from an escapable small, bendy nylon thread (Von Frey hair). A small incision of the skin and muscle of the underside on the animal's hind paw is then made and repaired under anaesthesia. One day after surgery, baseline paw withdrawal threshold from Von Frey hairs is then measured. Typically two days after surgery a novel treatment, standard analgesic (eg morphine), or vehicle is administered before the animal's paw withdrawal threshold from Von Frey hairs is measured again. The latency to withdrawal of the paw from a heat source may also be measured after dosing.

Blood samples may be taken from tail veins during the protocols, of after the animal has been killed.

It is expected that up to 480 animals will be tested each year under this licence.

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

In the case of naive animal tests, extremely few adverse effects are expected because pain sensitivity is measured using an escapable heat source, which is set at a level that will not harm the animals.

In the case of sensitivity following an incision, the rats may show signs of sensitivity in their paw upon stimulation but no signs of obvious 'background pain' or weight loss are expected. The animals may be kept for up to 5 days following surgery, during which time they will be monitored for signs of pain.

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

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

The vast majority of naive animals >90% used under this licence are expected to experience mild severity.

Animals that undergo incision surgery are expected to experience moderate severity as a result of the surgery.

All animals will be monitored, and will not experience beyond the moderate category of severity.

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

- Killed

Replacement

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

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

The in vivo models of pain described in this licence application are employed to generate information about baseline pain sensitivity and pain sensitivity following surgery in the context of the whole body after a novel treatment has been administered. It is neither possible, nor ethical, to use human volunteers in early drug discovery. It is therefore necessary to use other whole body systems, animals, to find out how a living organism responds.

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

The applicant has reviewed the literature, but has not been able to find any non-animal alternative models of post-operative pain.

During August 2023 Medline and Google Scholar were searched over the period 2017-2023, using the following relevant key words 'in vitro model of pain', 'refinement model of pain', 'in vitro model of pain replacement'.

Why were they not suitable?

No proposed non-animal models have been found to exist.

In general, proposed 'cellular models of pain' were identified which describe peripheral sensory neuron preparations. Although useful in the screening of compounds which act at specific peripheral targets, these types of models do not replace the need for in vivo testing. Peripheral sensory neurons are the initial transducers of noxious stimuli; however, they are only the first relay in the complex pain pathway, which involves diverse cell types, intricate spinal cord circuitry, a fine balance of ascending and descending neural traffic, and coordinated recruitment of many brain regions. The models covered by this licence allow the assessment of novel potential pain treatments on the complete integrated in vivo response to pain.

Reduction

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

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

The number of animals used in each study is generally chosen on the basis of previous experience in using the models and generating useable data. Also, for the protocols covered by this licence a professional statistician has been consulted to ensure an experimental design is optimal and minimises the number of animals required, yet ensures an adequate level of precision and power and the appropriate analysis is performed. Furthermore, where appropriate industry guidelines or current best practise will be followed. Design factors such as randomisation or level of replication will also be considered.

Group sizes will vary between protocols. In some experiments as few as 2 animals will be employed per group (e.g., dose finding pilot experiments). However, other experiments, such as a pain model procedure may typically require 6-10 animals per group. In some cases, for example when choosing between two development candidates, it may be necessary to determine drug effects more precisely. In this case the first consideration will be to conduct meta-analyses if deemed appropriate, but it may be necessary to replicate studies (thus increasing the number of animals per group) in order to look for smaller scientifically relevant differences or to obtain more precise estimates of treatment differences.

For a particular procedure, the magnitude of effect sizes of clinical interest are generally set after observing the size of response observed following administration of a clinically relevant control drug.

The number of animals per group is then ascribed for subsequent studies so that similar sized effects, if real, can be detected with adequate power.

As an estimate, it's reasonable to assume that in a given experiment a novel compound will typically be tested at 3 doses with 2 controls per experiment (a vehicle treated group and a positive and clinically relevant control) with 8 animals per group, and an average of 1 experiment will be carried out per month. Over the 5 year timeframe of the project, this would equate a total of 2,400 rodents.

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 per group and experimental design are determined on the basis of power analysis, advice from statisticians, published data and previous results that have consistently identified target effects in a clear and unambiguous manner.

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

Within each experiment a positive control (typically morphine) is included to provide an internal control to compare the relative efficacy of the test compound and to assess the sensitivity/validity of the test procedure on a given test occasion. This good experimental design principle will avoid unnecessary replication of experiments.

Initial pilot studies in small numbers of animals will be used to determine a dose of compound that can be administered safely and without inducing behavioural disruption or adverse events before progressing to studies that require larger numbers of animals. It is anticipated that the impact of this will be to reduce the overall number of animals used.

Where possible and appropriate, multiple end-points are taken including compound and biomarker level determination. This strategy increases the power of each study and reduces the potential requirement for future studies to assess cytokines exclusively.

Refinement

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

Which animal models and methods 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 tail flick test is a widely used test of nociception (perception of a painful stimulus). This is probably the most widely used of all nociceptive tests, and is used to define whether novel test agents have the ability to block the nervous transmission of normal pain signals.

In the mouse version of this test the animal is held and the tail dipped into water that has been warmed to 50oC and is contained in such a way that the animal can remove its tail.

In the rat version of this test radiant heat is focused on the tail, and the time is measured until the animal flicks the tail away from the beam.

As the animal can withdraw its tail from the heat source in both versions of the model, the heat stimulus is escapable and refined in the sense that the animal will not experience pain to the extent that it will become distressed. If analgesia is experienced by the animal to the extent that it does not feel heat, there is a time limit of exposure to the source to ensure no damage occurs the animal's tail. The time limit is 40 seconds in the mouse model, and 15 seconds in the rat model.

The hotplate test assesses nociceptive responses of rodents placed on a heated surface at 45-55oC, which is kept consistent within each experiment to match comparator historical protocols where necessary. The latency to various behavioural responses, including shaking of a foot and licking the fore paw, the hind paw or both, is used as a measure of pain sensitivity. Some versions of this model record the number of responses during a set period of time, but the version covered by this licence is refined in that, as soon as the animal displays a response to the heated surface it is removed and placed back in its home cage. If analgesia is experienced by the animal to the extent that it does not feel heat, there is a typically a 20-second time limit of exposure to the hotplate to ensure no damage occurs the animal's paws.

Developing novel drugs, requires the use of models that translate to the clinic in terms of face (same or similar measures), construct (comparable aetiology) and pharmacological (sensitivity to the same drug treatments) validity. Although the tail flick and hotplate models provide pharmacological validity, they do not provide face or construct validity for post operative pain. A post-surgical model is therefore also covered by this licence, which provides face and construct validity in addition to pharmacological validity. The Brennan model of post-incisional pain is one of the most commonly utilised of these models rats and has recently also been developed in mice. The model tracks the hyperalgesia and allodynia associated with an incision of the skin and muscle of the plantar surface of the rodent hind paw. The model is refined in that the animals do not display signs of ongoing background pain, and escapable hyperalgesia responses to mechanical or thermal stimulation are used to measure the efficacy of novel compounds. Furthermore, control and inspection protocols are in place to ensure that potential discomfort and distress are minimised.

Laboratory staff are trained in handling and ensuring the well-being of animals in their care. Training courses, organised by the NACWO and PPL holders, have been implemented to ensure that all practitioners are competent in the various dosing, handling

and surgical techniques. This includes internal competency tests for various techniques, which are signed off and dated by approved training staff or the NACWO.

Animals are euthanased as soon as possible after the end of a procedure.

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

Mice and rats are the lowest sentient species that are acceptable as capable of generating a pain response which models relevant aspects of postoperative pain in humans.

Mice and rats are very similar to humans physiologically and genetically, they're readily available, and they're straightforward to handle.

Whilst there are similarities between mice and rats, there are several physiological differences between the two that are considered when deciding which to use for a particular experiment.

There is no evidence for a difference in sentience between mice and rats, so this is not the basis for choosing between them for specific experiments.

Adult rats weigh up to ten times more than adult mice, which makes them the preferred species for applications that involve surgical procedures, including the Brennan model of postoperative model of pain covered by this licence. Surgery is generally easier in a larger animal, and causes less relative tissue damage. However, if previous experiments for a particular project have been carried out in mice and comparator data is required, mice will be used. For instance, if a particular project has previously carried out studies in a genetic mouse model under a different licence, direct comparison with wild type mice might be required in the models covered by this licence.

Compounds are typically tested under this licence initially in Protocol 1 (Determination of drug effects), before then being tested in Protocol 2 (Measurement of nociceptive thresholds). Typically, if active in Protocol 2, a compound may then progress to being tested in Protocol 3 (Model of Postoperative pain). As Protocol 3 is a surgical protocol, rats may be used more frequently than mice in this protocol for the reasons described above. To maintain a continuity of species throughout the testing of a particular compound, this means that rats are also more likely to be used than mice in the non-surgical Protocols 1 and 2.

Also, the smaller size of mice does offer some advantages, including requiring lower drug dosages, which makes them appropriate to use if there is a limited amount of compound available for testing.

The choice of mouse or rat may also depend upon pharmacological and/or pharmacokinetic resemblance of the chosen species to man for a particular project or class of chemical agent being tested.

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

Environmental enrichment for laboratory animals is actively pursued. Conditions in the animal house follow current best practice, and items such as tubes and red Perspex boxes are placed in rodent cages for their stimulation.

Laboratory staff are trained in handling and ensuring the well-being of animals in their care. Training courses, organised by the NACWO and PPL holders, have been implemented to ensure that all practitioners are competent in the various dosing, handling and surgical techniques. This includes internal competency tests for various techniques, which are signed off and dated by approved training staff or the NACWO.

Clear-cut end points are described in the possible adverse event description for the protocols covered by this licence. Advice notes and weight-gain sheets will be present in the holding room of animals.

Anyone can therefore ascertain instantly whether any animal is experiencing undue discomfort and distress, and know what to do if this occurs in order to minimise it, including a request for pain assessment via the grimace scale by the NACWO.

Postoperative animals will be weighed daily and monitored daily for pain and infection. A large number of possible signs of pain and infection will be assessed daily, and noted if observed (see 'Pain and Infection Monitoring Plan' provided as part of Step 1 in Protocol 3). Appropriate action will be taken if signs of pain, or infection are observed, and the animal will be killed by a schedule 1 method before severe signs are observed.

The size and frequency of blood samples taken will be limited so as not to cause haemodynamic fluctuations.

Animals are killed as soon as possible after the end of a procedure.

Sotocinal et al. (2011). The Rat Grimace Scale: A partially automated method for quantifying pain in the laboratory rat via facial expressions. Molecular Pain, 7:55

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow best practice guidance published by https://www.nc3rs.org.uk/

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I shall regularly review https://www.nc3rs.org.uk/, and carry out the literature searches described in the 'Replacement' section above every 6 months to identify applicable advances in the 3Rs. I will also maintain awarenes of relevant output from the Reading

University's AWERB, 3Rs Group, and general in-house distribution of 3Rs advances. Any appropriate advances will be implemented where appropriate.

In addition, experiments will be continually assessed for possible further refinement possibilities to ensure that the potential for discomfort and distress is minimised, and to ensure that they are conducted in a manner most likely to produce a satisfactory outcome.

40. Studies on the regulation of iron metabolism

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- 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

Iron, deficiency, anaemia, overload, chelators

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?

To determine what controls body iron levels and to evaluate new drugs for the treatment of iron-related 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?

Iron deficiency anaemia (IDA) is a global health issue affecting both developed and developing countries. IDA has a greater effect on population subsets where demand for

iron is higher than normal e.g., pregnant women and pre-school children. A recent report by the World Health Organization (WHO) estimated that the prevalence of IDA in preschool children in the Americas was 29.3 % affecting 23 million individuals and for pregnant women 24% with 3.9 million affected. In severe anaemia, there is an increased risk of mortality. There is evidence that even mild anaemia has effects on cognitive development in children and adults, fatigue and tiredness due to anemia lead to loss of productivity as well. Hence, understanding what regulates iron metabolism and dietary iron absorption and how to make interventions can have a significantly positive impact on healthcare for millions of individuals with IDA. Under our previous project licences, we have identified genes in the mouse which are directly involved in dietary iron absorption as well as others involved in human diseases such as the ferric reductase (Dcytb) and ferroportin, the protein that transports iron from the inside of a cell to the outside of the cell. The study will enable further understanding of how these genes and proteins are regulated. We are also interested, in identifying other important iron metabolism genes which may contribute to a better understanding of human diseases. In collaboration with our industrial partners, we hope to translate our basic research into new treatments for IDA as it is hoped that the novel iron compounds will advance to preclinical testing stages in humans. These new iron compounds are promising in addressing deleterious consequences of gastrointestinal disturbance that are associated with iron compounds that are currently used in the management of anaemia. The studies with mouse models will lead to a better understanding of the mechanisms underlying anaemia in whole animals and give further insights into how to manipulate iron metabolism for the greater benefit of anaemic patients. The development of novel iron compounds will advance efforts towards the treatment of iron deficiency.

What outputs do you think you will see at the end of this project?

The studies with mouse models will lead to a better understanding of the mechanisms underlying anaemia in whole animals and give further insights into how to manipulate iron metabolism for the greater benefit of anaemic patients. The development of novel iron compounds will advance efforts towards the treatment of iron deficiency in patients. The outcome of the study will be published in reputable journals and presented at the biennial BIOIRON Congress and the yearly European Iron Club.

Who or what will benefit from these outputs, and how?

Research output from the licence would potentially benefit several sectors, including the academic community, industry, policy makers, health practitioners as well as the public. Iron status has been highlighted as a public health concern in several population groups. Findings from the proposed studies will improve our understanding of optimal iron dosage, for efficient functional benefits. Long- term outputs from the project could include the synthesis and commercialization of novel iron compounds for the treatment of anaemia.

How will you look to maximise the outputs of this work?

The most immediate beneficiaries of the work would be researchers in the field of iron metabolism (UK and international) who would be informed through open-access publications in reputable journals.

Dissemination of the data generated from the project will also be at the attendance of national and international conferences. When required research from this project will be expanded through a range of national and international collaborations.

Results significant to public health will be communicated to the media and appropriate government departments by members of the project team through the institution's press and research office.

Species and numbers of animals expected to be used

- Mice: 500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Our overall research strategy involves cell culture, tissue culture, animal studies and studies in man. Cell culture is useful for investigating certain fundamental mechanisms at the cellular level, however available intestinal cell lines are of limited value as they are cancer cell lines and do not express all the relevant genes found in the duodenum of the small intestine (especially Dcytb, the enzyme required for dietary iron absorption in the duodenum of mammals). Iron metabolism, however, occurs in several complex tissues, such as the intestine, spleen, bone marrow and the liver. The regulation of iron metabolism involves interactions between tissues and between differing cell types within tissues which have yet to be understood. It is not yet possible to model these interactions in vitro hence in vivo animal work is a necessary part of understanding iron metabolism. We are conducting parallel studies with in vitro systems and in cultured cells and wherever feasible, the latter will be used. Any one of our studies involves at least three approaches, eg cell culture, tissue culture and animal work.

We will experimentally alter iron metabolism to enhance iron absorption in weaning mice that are approximately 3-week-old by feeding a purified iron-deficient diet for not more than 8 weeks. Weanling mice grow rapidly and because they will produce large numbers of red blood cells whilst being fed an iron-deficient diet, they become anaemic within 6 weeks. In other words, their haemoglobin (Hb) levels are depleted to create anaemic mice for iron repletion studies. It is a useful experimental intervention for investigating iron absorption by oral application of iron compounds (iron supplements), once daily for Hb repletion for 2 weeks.

Typically, what will be done to an animal used in your project?

Iron compounds will be synthesised and after initial screening in cell culture models of iron uptake, promising compounds will be tested in mice. Mice will undergo a well-established method known to alter iron metabolism, by feeding an iron-deficient diet for up to 8 weeks (control animals will receive a control/normal diet). In our experience, animals are iron-deficient after 6 weeks of being fed iron- deficient diet. Blood micro-samples may be taken up to 3 times during the 6 week period to check the level of Hb depletion and to randomly distribute the mice into experimental groups. Once sufficient iron deficiency is achieved (and at a maximum of 6 weeks), the animals may undergo one of the 2 experimental pathways. Mice that are not anaemic after 6 weeks of low-iron feeding will not be treated with iron supplements and may be excluded from the study. The 2 experimental pathways are as follows:

Pathway 1 - Iron Absorption testing (typically 25% of mice)

(1) For the first set of experiments on iron absorption, mice that have been fed either a normal/control or iron-deficient diet will be given radio-labelled iron compounds by oral gavage once and left for 4 h for iron absorption during this period. Oral gavage will be done only once to ensure the direct delivery of the radioactive iron compounds into the stomach of the mice. Absorption of radio-labelled iron compounds can be measured and tracked within a short duration such as a 4 h period. Blood and tissues will be collected after 4 h to analyze radioactivity to calculate iron absorption as a non-recovery procedure.

Pathway 2 - Hb Repletion (typically 75% of mice)

For the second set of experiments, iron-deficient mice will be given novel non-radioactive iron compounds, orally once by pipette or gavage, daily for 14 days for Hb repletion. Oral delivery by pipette or gavage ensures the delivery of the iron compounds into the stomach of the mice. A duration of 2 weeks is required for responses in Hb repletion in the mice. Mice will receive an iron-deficient diet for the duration of this experimental pathway. For Hb repletion studies, it may be necessary to collect faecal samples. This will be done during the last 24 h of the 14 days period of oral iron dosing. Mice will be housed in cages with absorbent bottom layered for the final 24 h of a treatment. Cages will be enriched with plastic rolls and shelters during this period. After treatments, mice are then killed to collect blood and harvest the tissues for analysis as a terminal procedure.

What are the expected impacts and/or adverse effects for the animals during your project?

None

Expected severity categories and the 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 are expected to experience 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?

Any one of our studies involves a first approach of cell culture to identify lead iron compounds (screening) before iron absorption studies are conducted in animals. Iron metabolism, however, occurs in several complex tissues such as the intestine, spleen, bone marrow and liver. The regulation of iron metabolism involves interactions between tissues and between differing cell types within tissues which have yet to be understood. It is not yet possible to model these interactions in vitro hence in vivo animal work is a necessary part of understanding iron metabolism.

Mice are the lowest vertebrate group which models human iron metabolism and iron disorders that occur in humans. The control of iron metabolism is determined by the interactions and communication between cells and tissues in the body. For example, the liver cells store iron and control the secretion of the hormone (hepcidin) which signals to the intestinal cells to release iron into the blood for uptake by the spleen and bone marrow.

It is currently not yet possible to investigate such complex body’s metabolism and interactions without using animals.

The factors which control differential iron uptake into tissues are not understood, nor are the various signals regulating the hormone (hepcidin) production. Information on human iron dynamics is also currently patchy. There is, therefore, little choice at present but to carry out measurements on whole animals.

Organoids will enable the study of how cells interact together in an organ and how they respond to various treatments such as different compounds and drugs in a way that mimic closely animal physiology. Organoids are three-dimensional tissue cultures grown from stem cells and embedded in Matrigel that support the cells. This in vitro system facilitates their monitoring and allows easy manipulation of several experimental variables that will streamline or reduce the number of animal experiments. Further research and characterization are required to define specific organoids that will mimic iron disorders. This is not currently available for iron absorption studies.

Which non-animal alternatives did you consider for use in this project?

Our overall research strategy involves cell culture, tissue culture, animal studies and studies in man. Cell culture is useful for investigating certain fundamental mechanisms at the cellular level, however available intestinal cell lines are of limited value as they are cancer cell lines and do not express all the relevant iron genes found in the absorptive surface of the gut cells) Cancer cells do not model normal gut cells. We are conducting parallel studies with in vitro systems in cultured cells and wherever feasible, the latter will be used.

Attempts to construct models of mouse iron metabolism remain rudimentary due to the lack of data on how these factors interact. Information on human iron dynamics is also patchy. There is therefore little choice at present but to carry out measurements on whole animals. More complex models using organoids or organ-on-a-chip models have not been found in the literature for iron absorption studies. This is because the regulation of iron metabolism involves interactions between different tissue types and the action of hepcidin, which is not produced under in vitro systems, which makes such in vitro models unsuitable for this project. During the duration of the project, we will seek non-animal alternatives continually for any part of the research. Online tools such as SyRF will be used to seek evidence on non-animal alternatives from systematic reviews and meta-analysis of animal studies.

Why were they not suitable?

Cells cannot adequately replicate the systemic interaction of proteins that are involved in iron metabolism in the liver, spleen bone marrow and gut. Informative computer simulations of iron metabolism are not yet possible as we do not have sufficient bioinformatic information on the proteins involved and how they respond to changes. cell cultures or computer models cannot adequately recapitulate the interactions between all the organs and tissues you require to study iron metabolism and regulation. Cultured cells, organoids or organ-on-a-chip models cannot recapitulate the studies on iron absorption because hepcidin, the hormone that regulates iron metabolism, is not produced in these systems hence, such models are not suitable to achieve the objectives of the proposed 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?

Usually, the experimental designs involve the comparison of different compounds or varying dose regimes. Our previous experience suggests that a group of six mice (to investigate the effects of two factors on an outcome) gives confidence and statistical

differences in the detection of biologically meaningful differences if these exist while using the minimum number of animals.

Five lots of six iron compounds are synthesized yearly to be tested and this makes 7 groups (1 control and 6 iron compounds) and with 6 mice per group, 42 mice will be required for each experiment. For iron absorption and Hb repletion studies, this is a total of 420 mice to evaluate the iron compounds. An additional 30 mice are required for attrition purposes, making a predicted total of 450 mice.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We use the NC3Rs' experimental design guidance and experimental design assistant (EDA) to plan our experimental design and support for randomisation and blinding, sample size calculations and appropriate statistical analysis methods.

Control mice are shared across different experiments to reduce numbers. We also collect tissues from the animals for other relevant research studies.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Information in respect to studies will be obtained from published literature and control animals that are not subjected to any treatment will be shared across experiments conducted at the same time, thereby avoiding duplication of animal numbers. We have used the types of models proposed in the current license previously and they have proven to be robust, producing large effects with a clear, reproducible phenotype. In vitro screening will be done to determine and streamline the number of compounds to be tested in mice. Information on iron compounds used to treat anaemia in humans will be searched in the literature.

After the experiment, all tissues are harvested from the carcass and kept frozen for use to address other relevant research questions by us or by other researchers working on related themes.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

CD1 mouse strain will be purchased and fed a low-iron diet to create an iron-deficient model. CD1 mice have been previously shown in our studies to be a robust and reproducible murine iron-deficient model. To induce iron deficiency to deplete Hb, animals will be given an iron-deficient diet to let us investigate how iron compounds will replete Hb levels in the mice.

Up to 6 weeks after being placed on an iron-deficient diet, iron absorption or Hb repletion studies will commence for 4 two weeks respectively. Some mice will require less than 6 weeks on an iron-deficient diet to become anaemic before the start of iron absorption or Hb repletion studies.

Blood sampling by tail bleeding will be done up to 3 times during 6 weeks on an iron-deficient diet to determine the level of Hb depletion in the anaemia condition. Only a drop of blood (5 µl) is required for the protocol. The depletion does not result in a clinical symptom but only allows measurable repletion with the tested iron compounds

(1) Study 1 - Iron absorption study: Oral once-gavage of radio-labelled iron compounds can be necessary to determine iron absorption within a short duration. The radioactive iron solution would not cause harm or toxicity but would give a robust reproducible study outcome. Iron compounds to be tested are intended as oral supplements for the treatment and prevention of iron deficiency in animals.

(2) Study 2 - Hb repletion study: Mice will be given the iron compounds orally daily for 14 days. Blood and tissues will be collected at the end of the experiment as a terminal procedure. Big cages will be used to collect faecal samples for microbiome analysis only for the last 24 h of the experimental protocol on Hb repletion. The use of cages (singly housed mouse), is preferred to metabolic cages for this period because animals are given respite such as environmental enrichment and tube rolls for play or for sleeping. Mice do not show any adverse effects in any of these protocols from our experience. There are no adverse effects with Hb depletion and iron treatments in the studies.

Why can’t you use animals that are less sentient?

No other alternative is translatable for models of anaemia in humans (e.g. Zebrafish do not simulate iron metabolism proteins and functions. Nor do they recapitulate the conditions of gastric duodenal effect on iron solubility and absorption which occur in mammals). Many of the important disease- causing proteins of iron metabolism were discovered in mice and were then found to do the same job in humans and were found to be defective in some human diseases. Therefore, mice are the lowest sentient animals most appropriate for our studies.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Administration of substances:

Some animals will be given substances by mouth or through food. Oral gavage given once is necessary to determine iron absorption over a 4 h duration. Oral gavage will be done only once for a radioactive iron solution to measure the amount that is absorbed from the dose administered. This allows the measurement of iron absorption during a 4 h period which is tolerable by the animals. Mice will be given the non-radioactive iron compounds orally daily for 14 days. Experience has shown that the administration of oral iron gavage is tolerable by animals.

Animals on an iron-deficient diet will be monitored for weight changes. Experience has shown that animals on a low iron diet do not show overt clinical symptoms even when fed an iron-deficient diet for up to 8 weeks.

Blood sampling to determine levels of anaemia induced:

Tail bleeding of animals (restrained in a tube) will be carried out up to 3 times during the initial 6 weeks of iron-deficient feeding. Only a drop of blood (~5 µl) is required at each time for up to 6 weeks of feeding. The ultimate aim is to identify iron deficiency as early as possible before further studies in this anaemia model.

Use of radioactively-labelled iron to understand how much of it is absorbed:

The dosage of radioisotope-labelled iron administered only once in the iron absorption studies does not cause adverse events. The use of low radioactive doses for a short duration of 4 h does not affect the normal physiology of the animal. However, radioactive iron allows the comparison and determination of iron that is absorbed from the different novel iron compounds and allows the tracing of the fate of the iron during the absorption process.

Use of cages with absorbent bottom layered to collect faeces for analysis: Animals will be kept in the cages for 24 h only. The cages are less stressful than using traditional metabolic cages because they allow environmental enrichment to be provided and therefore the provision of shelters for the animals. A 24 h faecal sample collection allows robust data generation without keeping animals in the cages for a longer period.

Use of iron-deficient diet to induce anaemia: Using diet as a method to create iron deficiency is a milder approach than other methods such as orbital bleeding.

The degree of anaemia that develops after feeding mice on an iron-deficient diet for up to 8 weeks does not cause any physical signs or reduce the activity of the mice. However, animals will be monitored daily and in the unlikely event that animals show signs of pallor of the ears, paws or other effects such lack of physical activity will lead to culling of the animals by a Schedule 1 method. In the unlikely event that mice show ill health or discomfort (e.g. lack of grooming, feeding or normal movement and social interactions in the cage, signs of infection etc) they will be killed by a Schedule 1 method.

Animals will be monitored daily. Any animal showing any distress will be culled.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

There is a robust availability of up-to-date information on websites on good practice, ethics, and animal welfare. There are published refined practices on oral dosing of mice on the website: https://doi.org/10.1258/0023677011911345

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I receive emails and check on animal welfare and alternative bodies that promote the principles of 3Rs, ethics, and animal husbandry. These include the LASA Institute of Laboratory Animal Research, NC3Rs, Understanding Animal Research, RSPCA, and Fund for Replacement of Animals in Medical Experiments (FRAME) amongst others. I check information on the NC3Rs website regularly and attend events such as the Regional 3Rs symposia. For example, I attended the Pan-London 3Rs Symposium on the 19th of April, 2023.

41. Development of gene and cell therapy for ocular and CNS conditions

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

Gene therapy, Stem cell therapy, Retinal degeneration, Neurodegeneration, Neovascularisation

Animal types	Life stages
Mice	adult, pregnant, embryo, neonate, juvenile
Rats	embryo, neonate, juvenile, adult, pregnant
Rabbits	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 the project is to develop novel treatments for neurodegenerative and neovascular conditions of the eye and 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?

Blindness due to retinal degeneration, especially the severe early-onset forms that we are focusing on, has a substantial impact on the quality of life of the patients. Childhood dementia caused by neurodegeneration leads to severe disability, including loss of vision, and early death in children. To date, there are no treatments available for these conditions. Ocular neovascularisation led to loss of vision and blindness in people of all ages and while treatment is available, it creates a very high burden for the patients and for society.

This project has the potential to cure or control these conditions and improve the quality of life of patients, as the previous project periods have led to therapies that are currently achieving promising outcomes in ongoing clinical trials.

What outputs do you think you will see at the end of this project?

At the end of the project there will be scientific publications describing the development of novel gene and stem cell therapies for neurodegenerative and neovascular conditions in the eye and brain, and there should be clinical studies ongoing that test these newly developed therapies in patients. In the long run, some of these should become established treatments that improve the quality-of-life of patients with sight-threatening conditions.

Who or what will benefit from these outputs, and how?

For the immediate duration of this project, the outputs will benefit the scientific community, with new information becoming available that will help them in their work creating other novel therapeutics. In the medium term, the work may benefit patients enrolled in clinical trials to assess therapeutic efficacy of the treatments developed in the project. In the long term, the outputs may benefit patients with a range of conditions if successful new therapies, developed as part of this project, aid in the design of further treatments.

How will you look to maximise the outputs of this work?

The development and testing of novel therapeutics is a major undertaking that requires input from a wide range of specialists. Collaboration is therefore an essential and natural aspect of this project. Our group works closely with collaborators in the UK and abroad, exchanging information, experience and materials to optimize the opportunities. Results, including disappointing ones, are discussed with collaborators, published in scientific journals and presented at international meetings. Engagement with patient organisations will be used to create wider awareness of our work, especially when clinical trials are in the pipeline. Clinical trials must be registered at official databases such as clinicaltrials.gov, ensuring comprehensive reporting of results takes place, irrespective of the success of the outcome.

Species and numbers of animals expected to be used.

- Mice: 10000
- Rats: 100
- Rabbits: 150

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

As the least-advanced animal with a retinal structure that is similar to the human retina, most studies will be using juvenile or adult mice, either wildtype animals, or animals that have an inherited disease. For the assessment of treatment for very severe neurodegenerations, newborn animals may be used to ensure treatment takes effect before there is extensive loss of neurons. Occasionally, juvenile or adult rats or adult rabbits may be used as the larger eye will allow a more precise surgery. Before patients can be treated, the safety of new therapies needs to be tested in more than one animal species; to this end, we will use mice and rabbits.

Typically, what will be done to an animal used in your project?

Animals will receive an injection of a therapeutic agent (usually a gene therapy vector or a cell- transplant) or a sham control injection at the site of the pathology (typically the eye or the brain). Generally, this would be a single administration without the need for repeated dosing. As the disease progresses, efficacy of the treatment is assessed periodically (using measures of retinal function or structure, vision, mobility, behaviour). The duration of each study is dependent on the natural rate of functional decline in the disease that is being treated and may vary from several weeks to over a year. Animals will not be sedated more than 30 times over the course of the study. At various time points after treatment, animals will be humanely killed to determine with greater precision the progression of the disease and the effects that the treatment had.

What are the expected impacts and/or adverse effects for the animals during your project?

A variety of natural and artificial mouse strains with inherited blindness exist. Because rodents are nocturnal animals who mostly use other senses (smell, touch), the blindness has little impact on the animals' welfare. The animals with forms of childhood dementia can develop problems such as paralysis and shaking. We ensure that animals are not kept alive when these problems start to become apparent to avoid the animals suffering.

The new treatments will be administered into the brain or in one eye of the animal, while it is under anaesthetic, with pain-relief and antibiotic/steroid ointment to further minimize any discomfort. When the treatment has taken effect, we can determine how well the brain or eye in the animal is functioning. These functional tests do not require any surgery and are designed to have minimal impact on the wellbeing of the animal. The duration of each study will to a large extent be determined by the innate rate of neuronal degeneration in that animal model. In a severe model with rapid vision loss, a study may take 2 or 3 months, while a study in a model with very slow degeneration may last up to 15 months. Other than the disease phenotype, no general abnormal effects are expected.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice: 40% of animals will be sub-threshold, 30% of animals will be mild, 30% of animals will be moderate.

Rat: 50% of animals will be mild, 50% of animals will be moderate Rabbit: 70% of animals

will be mild, 30% of animals will be moderate.
What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The aim of the project is to improve vision, mobility, cognitive function and survival through gene therapy or transplantation of neuronal cells. In order to do so, we need to assess whether therapy has improved the function of the neurons, whether transplanted neurons survive and connect up with the existing network, whether the treatment elicits immune responses from the host, and most importantly, whether the therapy results in an improvement of behaviour (vision, mobility, etc).

Which non-animal alternatives did you consider for use in this project?

Cell lines in culture, including 661W photoreceptor cells, RGC-5 ganglion cells and ARPE19 retinal pigment epithelial cells, and stem cell derived retinal organoids in culture, to assess the effect of gene replacement on neuronal cell function. Use of microfluidics to assess the ability of stem cell derived photoreceptors to make synaptic connections.

Why were they not suitable?

Although our new treatments are tested in cultured cells prior to use in animals, the treatment effect can only be proven in animals, as the diseases we aim to treat are complex disorders, involving communication between multiple cell types, as well as the blood supply and the immune system. The putative therapeutic benefit of the treatment is derived from an interaction between the novel therapeutic agent and the whole recipient organism.

Current knowledge and techniques are insufficient to model all these interactions, either in a dish or using computers, well enough to reliably predict treatment outcomes.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Currently, we maintain around 20 strains of transgenic mice, either strains carrying disease-causing mutations, or strains that have a marker gene, usually expressed in specific neuronal cell types.

Keeping these lines breeding produces around 2000 animals a year, which are all part of the study, even if not all animals will be used in experimental procedures. We are currently investigating new treatment strategies for 10 different eye and brain diseases, running around 30 therapeutic experiments a year. These experimental studies typically use between 20 and 40 animals depending on the length of the study and the expected therapeutic effect.

Rat and rabbit studies use on average 25 animals per study and we do not expect to perform more than 4 studies with rats and 6 studies with rabbits over the course of the project.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

A statistician is available for group members to advise and assist in the design of experiments. Randomisation of animals/eyes, and masking of researchers are encouraged to improve the scientific validity of outcomes.

For a novel therapy, we will typically run a pilot experiment prior to a full-scale study to test whether there is likely to be a therapeutic benefit, and its approximate size and variability to inform statistical study design. Group sizes are based on Power calculations using the minimal clinically-relevant therapeutic effect and variability of assessments (known or estimated in pilot studies). As most assessment methods cause mild discomfort at most, care is taken to combine multiple (compatible) assessment methods in the most appropriate fashion to achieve the maximal amount of data from each experimental group. When multiple assessments are performed, group sizes are determined by the assessment requiring the largest groups.

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 stem cell transplantation project we have developed methods that enable us to grow human retinas (light sensitive structures from the eye) in dishes. Using them, we can test some of the effects of the new therapies on the light sensitive cells without using animals. We always try to improve our testing methodology to achieve greater accuracy, allowing smaller group sizes, e.g., by the recent purchase of electroretinography equipment specific for use in small animals.

Increasingly, in vivo assessments of retinal structure and function, including various methods to assess visual function, allow monitoring of disease progression in vivo rather than in post-mortem retinal tissue, thereby reducing the number of animals used since any given animal can be used for a number of outcome measures, and cross-correlation of various outcomes can strengthen the validity of data sets.

Ex-breeders that have suffered no more than mild discomfort during the course of breeding procedure may be re-used in other protocols of this licence 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.

As therapeutic studies for inherited diseases require a genetic animal model of disease, most studies will be performed in transgenic or natural-mutant mice. Because rodents are nocturnal animals who mostly use other senses (smell, touch), the blindness has little impact on the animals' welfare. The animals with forms of childhood dementia can develop problems such as paralysis and shaking. We ensure that animals are not kept alive when these problems start to become apparent to avoid the animals suffering.

Administration of the therapeutic agent is the least refined step in the methodology, but administration is unavoidable for the testing of a new treatment. Care is taken with analgesia/anaesthesia and post-operative use of ointment after all surgeries to minimise impact on wellbeing. Subsequent assessments of function only cause mild discomfort.

Why can't you use animals that are less sentient?

The structure of the eye and brain in non-vertebrate animals differs substantially from vertebrates, which makes them unsuitable for these therapeutic studies. Rodents are among the lowest vertebrate species with a retina that does not self-repair (comparable to human), which is important when assessing retinal treatments.
Some invasive assessments will be performed in terminally anaesthetised animals; however, that severely limits the number of assessments of function that can be performed per animal, thus substantially increasing the number of animals required for the studies. Most assessments that are performed only cause mild discomfort, making the use of terminal anaesthesia at the cost of greater numbers of animals counter-productive.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Procedures are continually refined to limit the negative impact on the animals. New methodology is explored as it becomes available, and adopted if deemed more refined (and appropriate for the task) than existing methods, such as the adoption of automated optomotor assessment of vision in favour of the forced-swim test used previously, or our recent development of a method that uses an innate freezing response to assess vision removing the need for training to noxious stimuli. Over the years analgesic/steroid care after administration of therapeutics has developed to a standard that adverse events such as ocular infections are very rare. For animal lines that are known to develop health problems (most notably the childhood dementia strains) extra health monitoring and strict humane endpoints are in place.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

NC3R is the main source of guidance used. The majority of animal models and procedures cause only mild discomfort at most. For the models that may experience moderate discomfort, most notably the more severe models of childhood dementia, we use score sheets and increased health monitoring to ensure humane endpoints are recognized and

adhered to in a timely manner. After therapy of slow- advancing models of disease, it may be necessary to allow animals to remain on protocol beyond 12 months of age. Ageing animals will be monitored more closely to ensure any health issues are recognized and treated appropriately after discussion with the NVS, or if necessary the animal will killed using a humane method.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I use the NC3R resource library and several members of my research group subscribe to the NC3R newsletter to stay up to date. The active users of laboratory animals in my group meet monthly specifically to discuss the ongoing and planned animal work, bring up practical issues they have encountered and suggest ideas for refinement of experimental procedures.

42. Evaluating Solid Dose Vaccination Technologies in Pigs

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Vaccination, Techniques, Immune response

Animal types	Life stages
Pigs	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To improve the efficacy of an immunization technique, which involves a needle-free method of delivering solid dose vaccines (SDV) into the subject's skin, by evaluating the effect of injection depth, and SDV composition on the immune response in pigs. The effect of *any* technical improvement is likely to have major potential benefits for large-scale immunization programs in both human and veterinary medicine.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Vaccination is one of the most cost-effective healthcare interventions and prevents millions

of deaths worldwide every year. Most vaccines require refrigeration after production and before use. They are delivered via a needle and syringe. The need for refrigeration significantly increases costs and wastage during distribution. It also limits vaccine availability in (typically tropical) areas where refrigeration facilities may be unreliable. The dislike of needles amongst the general population renders many vaccination programs incomplete, due to poor voluntary uptake.

Immunization attempts to increase an animal or human's resistance to disease - usually caused by bacteria or viruses - by boosting the host's immune response.. This is done by injecting vaccines. These contain non-harmful parts of the bacteria or virus (antigens) which improve the immune systems capacity to recognize and kill invading microbes. The effectiveness of vaccines, and their duration of effect, depend on numerous factors including the ability of the vaccine to provoke strong and sustained immune responses. This depends on the type of antigen used and how it is delivered into the body.

The traditional immunization of humans and animals has involved the use of hypodermic needles and syringes to inject liquid suspensions of the antigen. This may be painful (particular in children) and the effects short-lasting, requiring unwelcome re-dosing at regular, vaccine-dependent intervals.
Using pigs, the intended study aims to identify the form of SDV and its injection depth that produces the greatest immune response. This will reduce the stress of injection - and repeated injections - by reducing the need for re-vaccinations. This will also reduce the time expenditure of vaccination teams protecting humans or animal populations

What outputs do you think you will see at the end of this project?

New information will be obtained on the injection depth and the form of the SDV that promotes the greatest and most sustained immune response.

Products. The project will assist the improvement of a commercial needle-free solid dose delivery platform for the human and veterinary vaccination markets.

Who or what will benefit from these outputs, and how?

Successful study completion will provide supportive evidence that the company is targeting and achieving a delivery depth that will produce the desired immune response, progressing vaccines of interest to human clinical trials with a view to eventual commercialisation. Several vaccines of interest are being formulated by the company into its solid dose form in parallel to this testing. These are currently undergoing in vivo testing in appropriate animal models (under different license) before progressing to first-in-human clinical trials. All elements of the study will have potential benefits in animal health where large-scale single-time vaccinations are desirable, for example, in the pig and poultry industries, and in aquaculture.

How will you look to maximise the outputs of this work?

The knowledge will be used to improve the effectiveness of the vaccine delivery method and will be used to collaborate with vaccine partners to aid the development of solid dose thermostable vaccines for use in humans and animals.

Species and numbers of animals expected to be used.

- Pigs: 65 pigs

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Juvenile to adult pigs are to be studied because: i) it is universally held, and on strong scientific grounds, that pig and human skin share many structural and functional similarities; ii) the technology is likely to be used in pig vaccination programs in the long-term

Typically, what will be done to an animal used in your project?

There will be two types of animal experience on this study: i) non-recovery; and ii) recovery.

The (few) pigs in the non-recovery group will be terminally anaesthetised. Typically, pigs will be loaded onto an approved animal trailer and transported by road (approximately 10 - 30 minutes) to the facility. Upon arrival, pigs will be briefly examined (physically) and then sedated using drugs given by a single intramuscular (IM) injection, i.e., into a muscle. This will typically produce sufficient sedation for the animal to be anaesthetized without stress. Once they are unconscious they will be looked after continuously by trained veterinarians. Additional doses of rapidly-acting anaesthetic will be given if the animals start to recover. Whilst anaesthetized, each animal will be injected with up to 24 SDVs. (Each SDV is 4 mm long and 1 mm in diameter). CT scans will then be carried out until the SDVs have disintegrated and are no longer visible on the CT scan - and at most up to 8 hours after anaesthesia began. The animal will then be killed whilst anaesthetized and not released for post mortem examination, or disposal, until physical examination confirms the animal is dead.

The (majority) of animals assigned to recovery procedures will be similarly sourced and transported in groups of up to 5 animals. Upon arrival at the facility the animals will be group housed and acclimatized for 5 - 7 days. During this time they will be habituated to the humans looking after them by the random award of food rewards. They will also become used to handling and physical examination, including having their temperatures taken. Their health will be monitored daily. On the day of study, pigs will be sedated by IM injection. When adequate conditions are present, the auricular vein will be cannulated and a blood sample obtained (day 0). During anaesthesia (as described above) each pig will be injected with a single SDV into the skin and in a proportion of animals, a single CT scan will be performed. The animal will then be allowed to recover from anaesthesia in a strawed pen. The animal will be continuously observed until standing and after that, observed regularly and frequently until drinking, feeding, rising and movement without signs of discomfort are present. On days 3, 7, 14, 21, 28 and 35, blood sampling (typically, no more than 1 mL blood) will be carried out. If required, pigs will be sedated and, or anaesthetized with the intent of ongoing experimental refinement. On day 35 (or less commonly day 90) pigs will be euthanatized (using anaesthetic overdose given by vein) once the final blood sample has been collected.

What are the expected impacts and/or adverse effects for the animals during your project?

For non-recovery pigs, the greatest adversity will be a single intramuscular injection of pre-anaesthetic medication/sedative for the recovery animals, 6-7 similar injections may be faced over the following 35 - 90 days, though alternative routes of drug administration may be sought.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Up to five pigs will be used in non-recovery studies.

The majority will be allowed to recovery after at least one - perhaps up to six general anaesthetics - but the nature of the study means the severity band is moderate.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The beneficial immune response to the SDV will depend in great measure on the rate it will disintegrate once injected. This disintegration rate depends on numerous factors only found in living skin, e.g., a pulsating blood supply, warmth, normal biochemical conditions, the presence of living active inflammatory and immune cells. These factors are not found in dead animals.

Which non-animal alternatives did you consider for use in this project?

Synthetic skin phantoms and dead pigs have previously been used to test the delivery device (injector) but even these options have different characteristics to the skin of a living (anaesthetized) pig.

Why were they not suitable?

They do not reliably mimic human skin in terms of dynamic penetration properties, circulatory fluids or immune response.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Up to 5 animals will be required for the non-recovery studies. The first animal will be used to set a disintegration time baseline to evaluate the current SDV formulations of interest. Optimisation may be required thereafter necessitating the use of a further animal. A third, fourth or even fifth animal may be required at a later date to investigate pulsatile / sustained release formulations, but these will be combined with the other studies where possible.

- Up to sixty pigs will be used in recovery studies.
- Up to thirty pigs will be used to determine the effects of SDV characteristics, i.e., dose on the immune response*
- Up to thirty pigs will be used to determine the effects of one of three SDV implantation depths on the immune response*

*The "immune response" has two quantitative components: antibody levels and T-cellular responses.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The study has been phased and has already progressed through the use of i) synthetic skin; ii) cadaveric imaging studies. The terminal studies will involve pigs receiving the maximum number of test implants that will still allow scientific interpretation, i.e. Pig 1 = 24 SDVs; Pig 2 = 24 SDVs; Pig 3 = 16 SDVs. Pigs in the recovery studies will receive a single implant. The considerable variability in immune response to fixed challenges justifies the size of the animal groups proposed.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The cadaveric studies (already completed) were conducted on suitably sized animals killed after the completion of other studies. The bodies of the animals killed on the proposed study will be made available for further cadaveric studies, tissue collection (for research or educational purpose) or for veterinary educational purposes (anatomical, pathological, surgical).

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 juvenile to adult pigs of either sex in this study. The procedures planned, i.e. general anaesthesia, the implantation (under anaesthesia) of a 4 X 1 mm implant followed by CT scanning, are not likely - or do not - cause pain.

Pigs are chosen over less sentient animals because of the similar properties of porcine and human skin.

Why can't you use animals that are less sentient?

Given that the pig is an ideal dermatological model, the study will be staged, with preliminary work using terminally anaesthetized pigs. If successful, adult pigs will be used because an expectedly representative (of human) inflammatory and immune response will be required. These are unlikely to be developed in very young pigs.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animal husbandry, surgery and anaesthesia will take place at our establishment's facility. For recovery studies, acclimatization, habituation and socialization will be the norm. Pig anaesthesia will be supervised by the applicant or his designated representatives. Daily welfare checks will be carried out by the NACWOs or trained PIL holders. A study-specific report form will be designed - and used - to draw focus to (the very few) specific concerns with this study. Individuals involved with the study will meet at least once weekly to identify, discuss and implement experimental refinements, if and when they are considered necessary.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will refer to the PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) during the planning stages and report our studies according to the ARRIVE 2010 guidelines (Animal Research: Reporting of In Vivo Experiments). The principles laid out in these resources will enable us to design and conduct the experiment in the most refined way.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The NC3Rs website (https://www.nc3rs.org.uk/) will be consulted and full advantage taken of the annual seminar day organized by the Establishment's Services to identify changes in best practice and methods to improve animal welfare.
I receive newsletter and information updates from the NC3Rs, Understanding Animal Research; The British Laboratory Animal Veterinary Association, The Laboratory Animal Science Association, FELASA, the Nuffield Council on Bioethics, NOECOPA, and the RSPCA (Animals in Science Department).

Implementation of new ideas will occur through consultation with the NVS.

43. Inflammation in arterial disease and co- morbidities

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Heart disease, Inflammation, Co-morbidities, Therapy

Animal types	Life stages
Mice	adult, aged, 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 identify changes in inflammation in blood vessels in heart disease and related diseases, and, to assess potential treatments.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

To understand the basis for overactive inflammation in vessel walls which can cause or exacerbate disease and to develop new therapies.

What outputs do you think you will see at the end of this project?

Outputs will be new information on inflammatory mechanisms of arterial disease including in the context of co-morbidities. This new information will be summarised in publications and presentations to scientific and lay audiences. The outputs may, in due course, lead to the development of new therapies.

Who or what will benefit from these outputs, and how?

In the short term, outputs will benefit the scientific field by describing new inflammatory pathways that contribute to arterial disease including new molecules.
In the medium term, outputs will benefit clinicians conducting patient facing research (and patients themselves) if the basic research identifies existing treatments that could be repurposed for disease modulation and patient benefit.

In the longer term, outputs describing novel pathways may benefit patients (including with comorbidities) if new classes of drugs are developed.

How will you look to maximise the outputs of this work?

This project will include a wide network of collaborators. This is required to address the common co- morbidities associated with arterial disease e.g., infection or hypoglycaemia. New knowledge including unsuccessful approaches will be disseminated by abstract and oral presentation at conferences, student dissertations published on line after completion and journal publications including video submissions as appropriate and methods papers. We will make any novel strains generated, technical know-how and original data available to other researchers.

Species and numbers of animals expected to be used.

- Mice: WT 7000 GA 7000, this includes the breeding protocols. Statistical aspects principles within the application are generic but within each protocol there is a detailed description of how sample sizes and experimental design will be addressed.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 defined mice provide a reproducible system for conducting medical research with similarities to humans in terms of anatomy, physiology and genetics. Mice are susceptible to many of the same diseases as humans or these can be induced in adult mice in a standardised manner for research studies.

The project will use adult animals as the disease and comorbidities being studied start to develop at this time and continue throughout the lifespan.

Typically, what will be done to an animal used in your project?

Typically, mice will be housed in family groups with free access to food and water.
For monitoring of disease by infrequent microsampling (most procedures), mice will experience mild transient pain but no lasting harm. In relation to interventions (at least 50% of procedures), animals will experience mild, transient pain but no lasting harm from administration of substances. Examples include: treatments including nucleic acids, antibiotics, contrast agents (including gases), bacteria, inducing agents (non-harmful modified viruses carrying certain cargos) by injection, typically using standard routes (e.g. intravenous, subcutaneous, intraperitoneal, intranasal). Where efficient delivery of treatments is required (a subset of procedures e.g. genetic deletion or restoration of the

microbiome), gavage may be used. Where administration is required for prolonged periods (10% of procedures), animals will be surgically implanted with a pin port device or a slow release devices such as a mini-pump. Animals will experience some discomfort after surgery and mild to moderate pain which will be treated with analgesics.

During 30 minute surgeries (10% of procedures), under general anaesthetic, surface arteries may be tied off with a suture to study arterial thickening which follows over the next 2-4 weeks. Animals will experience some discomfort after surgery and some mild to moderate pain which will be treated with analgesics.
Mice may be fed altered diets similar in fat content to those consumed by humans in Western Society (at least 70-80% of procedures). Some treatments may also be administered with the diets. We may also use interventions that affect the gut microflora (the microbiome), 10% of all procedures.
These interventions and diet changes are not expected to cause distress but may sometimes result in obesity or itchy skin. Some diets may result in weight loss due to unpalatability. Animals will be placed onto normal diet should they lose 20% of their body weight. Older mice may be studied, these mice may be more frail than mice at middle age and will be assisted with feeding if this is required.

Mice may receive certain bacterial strains for example by an intranasal route to mimic the situation in humans when a respiratory infection occurs (max 5% of procedures). Mice are able to clear these bacteria quickly (in health), less quickly in vascular disease. Mice will experience minimal discomfort and will be closely monitored for bacterial clearance through blood cultures and general health an wellbeing. It may be necessary to mimic infection with certain substances found in bacterial cell walls or released by bacteria instead of bacteria to assess the effect of this comorbidity on vascular disease. Mice will experience minimal discomfort and as in human settings antibiotics may be given by standard routes.

Mice may receive contrast agents to assist with imaging (1-3% of procedures) for example hyperpolarised gases e.g. hyperpolarised Xenon, by inhalation, or injectable agents to identify certain types of cells or tissue accumulation e.g. radiolabelled tracers (similar to those used in humans). Mice will experience minimal discomfort.

The final procedures (100%) will be undertaken under non-recovery anaesthesia where the animals will only be aware of the anaesthetic being administered and may experience mild distress and no pain.

What are the expected impacts and/or adverse effects for the animals during your project?

Mice will receive Western-type diets to accelerate the development of arterial thickening. These can lead to greasy fur and itchy skin after extended periods of feeding (12 weeks). Mice can be switched back to normal chow if required to allow healing or individual lesion can be treated.

In extensive arterial (atherosclerotic) disease or in older animals with arterial disease, animals may show altered behaviour e.g. less interest in their surroundings. This may occur in the final month of a 6 month study.

Mice will receive some treatments through a tube leading down the throat into the stomach, called gavage. This causes temporary distress of 1-2 minutes whilst the procedure is being done. There are no longer lasting effects from this procedure.

Mice will 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 similar to humans.

Mice will have surgery to permanently tie off a particular artery with a suture. The mice recover quickly and will be given painkillers and post operative care. There are no long lasting effects.

Mice with comorbidities (no more than 3) may reach human endpoints earlier than mice with a single condition. We have developed clear guidance for use in these settings.

Older mice (18-24 months) may exhibit frailty which includes decreased body condition and weight loss. As with humans, extra care with feeding and detailed daily monitoring will be used. Clear guidance on humane endpoints will be used.

Expected severity categories and the 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 10% mild and 90% sub-threshold. Vascular Injury of GA mice 80% Moderate, 20% mild.

Atherosclerosis and co-morbidities in GA mice 75% moderate, 15% mild, 10% sub-threshold

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We need to use animal models to understand how arterial disease develops in vessels in a whole organism similar to humans. Arterial disease develops slowly in arteries that are very complex structures with many different cell types interacting and communicating with each other. We also wish to study other interacting co-morbidities such as certain types of infection, the microbiome present and the effects of altered glucose. These processes are so complex that currently no in vitro system is able to replicate this.

Which non-animal alternatives did you consider for use in this project?

In vitro models: these have been fully considered with data available from the group in question on inflammation in vascular cell types including smooth muscle cells, endothelial cells and macrophages and in human tissues e.g. organ culture.
Atherosclerosis research includes quantitative analysis of lesion size and plaque

composition which cannot be replicated in a dish in a laboratory. Animal research can provide experimental tissues and cells needed for detailed in vitro experiments at the various stages of the disease process.

Human vessels: to be accessible in sufficient quantity these are usually obtained at the end stage of disease so we cannot study the disease process as it develops. We have published on these in the past and recently worked with carotid endartectomy samples from humans to verify some of our findings in mice.

Organoid models: models of human arteries being developed but developing a blood supply is the achilles heel of all of these and thus far stem cell organoids have to be grown under the kidney capsule in a mouse. Newly funded work (2022) seems promising **https://www.nc3rs.org.uk/our- portfolio/development-and-disease-modelling-engineered-vascular-tissues** but is based on a fibrin-based 3D culture of primary human vascular SMCs only whereas the vascular wall contains at least 10 more cell types.

For many of the experiments proposed, our *in vitro* data with human cells inform the animal work e.g. with regard to dosing of a treatment.

Publicly available human, cell and genomic data: we use a variety of datasets to determine whether our hypotheses are unique and likely to be true including looking at genetic variation and polymorphisms in humans to inform our use of genetically altered mice.

Non-protected species such as fruit flies or nematodes are not suitable for studying physiological mechanisms of inflammation in diseased arteries.

Why were they not suitable?

Arterial disease is a chronic inflammatory disorder involving cells of the vessel wall and cells of the immune system. The disease is heavily influenced by plasma lipoproteins (cholesterol forms) in the blood and the hemodynamics of the blood flow in the artery.

Although *in vitro* models are suitable up to a point and can model isolated aspects of the arterial disease process, they do not capture the physiology, the blood flow (blood pressure) and other related factors that occur in a whole organism.

In addition, non-animal alternatives do not allow the study of co-morbidities where diseases occur together or allow the study of different organs in a disease process e.g. alteration of the microbiome, the study of arterial disease and any pathology occurring in 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?

We will use male and female mice for our studies.

All personal licence holders are trained in statistics as part of their higher degree studies.

In addition, calculations using typical variations from our own earlier published work were used to calculate minimum numbers of animals to generate statistically significant data. Sample sizes for experiments are estimated from past experiments, from the relevant literature or by direct contact with the research groups undertaking the specific procedure. For atherosclerosis endpoints, calculations typically show that we need group sizes of 12-16 (depending on gender of the animals) to achieve the quality data required.

The annual return of procedures data was used to estimate the number of animals that we will need to use for breeding. We will use male and female mice for our studies. We use a number of different strains and breeding procedures with Cre strains to genetically delete specific proteins that are implicated in arterial disease.

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 guidance and experimental design assistant (EDA) for experimental design, practical steps and statistical analysis utilising the advice and support for randomisation and blinding, sample size calculations and appropriate statistical analysis methods. We will use the EDA diagram and report outputs to support grant applications and experimental planning with animal users.

Mice will be ear clipped, randomised (housed together if possible) and assigned to groups by an individual not undertaking the experimental analyses. Experimental analyses will be completed in a blinded manner.
Control groups for surgical studies: for carotid ligation, we use the contralateral artery as a sham control.

For cre studies: we will use tamoxifen treated Cre negative animals as well as Cre-/+ vehicle treated animals as well as test groups as defined in PMID: 29336844
Efficient methods of gene deletion will be used and if necessary, these will be piloted to check the level of recombination in the tissues of interest.

Pilot studies developed with the NACWO and named vet will be used by any new PIL holder or any newly developed procedure, especially to set dosing schedules or with new comorbidity models where physical or cognitive tasks are required to be completed or humane endpoints need to be clearly defined. There are many advantages of conducting pilot studies as per **https://www.nc3rs.org.uk/3rs- resources/conducting-pilot-study**

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 as above.

Preparing tissue in as many ways as possible e.g. taking bone marrow, tissue for wax embedding and frozen sections as well as for RNA analysis.

Tissue sharing - at the end of the experiment, we will harvest as many tissues as possible at post- mortem and make them 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 366inimize welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 method of generating arterial disease that involves a single injection of an agent that leads to raised cholesterol in blood if the mice consume a Western diet. This is a new method which generates mild atherosclerosis leading to fewer skin lesions and which more closely mimics the human situation and can be combined with other comorbidities e.g. infection, amyloid accumulation or altered glucose.

Aged animals can be purchased, arterial disease commenced and comorbidities such as hearing or cognitive decline can be studied in the ageing animal. We will perform tests such as grip strength which is also performed in older humans.
To induce gene expression in animals or to deplete specific cells, 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 will have to induce changes in the gut microbiome to study this in atherosclerosis. Animals will be infected via the nasal passages similar to humans.

We will undertake imaging of our animals to assess cardiac function in a manner similar to humans either using an external probe or in an MRI scanner. We will use similar contrast agents to those used in man including using hyperpolarised gases to image arterial vasculature.

Why can’t you use animals that are less sentient?

Non-mammalian animals are limited in their use because their vascular system is too different and we know blood flow patterns are important in developing disease or they not have the right type of immune cell or their immune system is too different from the human immune system to allow us to study inflammatory mechanisms and potential treatments.

We cannot use embryos or very young animals as their blood vessels are too small, we cannot induce atherosclerosis by diet and their immune system is immature.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Monitoring: a frequent monitoring regime will be used with 'traffic light' score sheets developed for certain procedures.

Example: Mice with extensive atherosclerosis (those on a high fat diet for > 20 weeks) will be monitored for signs of declining coat condition in particular or a increase in aggressive behaviour. Single housing will be avoided if possible. If small skin lesions appear, these

can be treated (e.g. with clay) but if they do not decrease in size or number within 10 days then a humane endpoint will have been reached.

Example: Aged animals will be carefully monitored using a scoring system developed with the NACWO and named vet for the specific experiment being undertaken.

Group sizes will be increased to accommodate for loss of animals and to avoid single housing. Longer drinking spouts will be used, mash will be given and animals will be monitored for adverse effects such as changes in weight, dermatitis, piloerection, paleness, changes in mobility, lumps, eye defects, abnormal respiration, or stools. If these are observed animals will be treated accordingly, and animals that develop severe effects will be humanely killed.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We already follow the ARRIVE guidelines [PMID: 20613859] for reporting and will also adopt welfare guidelines set out in PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence).
In addition, we will adopt best practice on Cre strains to ensure veracity of breeding and correct use of controls using the NC3Rs guidance on breeding and colony management [https://nc3rs.org.uk/3rs- resources/breeding-and-colony-management/breeding-and-colony-management-faqs]

For in vivo imaging, we will view the latest literature on MRI of rodents (no literature is available at N3CRs other than for NHP imaging] particularly for arterial spin labelling [PMID: 34822744] and other MR techniques where these link to atherosclerosis associated pathologies.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Information on NC3Rs website will be checked frequently alongside theNC3Rs newsletter. PIL holders will attend conferences and seek out 3Rs solutions and if appropriate, implement these during the project.

44. Investigating respiratory distress in poultry

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes

Key words

Breathlessness, Animal Welfare, Poultry, Slaughter, Air hunger

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 aim of the project is to determine whether birds experience respiratory distress, so called ‘air hunger’, during exposure to gas mixtures relevant to controlled atmospheric stunning (CAS) used for commercial slaughter.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Globally, 72.4 billion chickens are slaughtered each year for food production. In 2021, in the United Kingdom (UK), 1.15 billion broiler chickens (reared for meat) were slaughtered across 50 operating slaughterhouses and 57 million spent hens (reared for egg-laying) were killed at the end of their production cycle. Poultry production represents the largest number of individual animals killed by humans for food production (excluding fish) by a large margin. As a result, the methods used to stun and slaughter poultry are critically important to ensure animal welfare standards for the most numerous terrestrial production animal.

There are two main methods of stunning chickens: electrical stunning and controlled atmosphere stunning (CAS). CAS is the primary method for poultry in the UK (~70%) and it is increasingly in use in Europe (EU) (~40%). CAS involves exposing chickens to gas mixtures, leading to unconsciousness and eventual death. CAS was originally developed to combat welfare harms associated with electrical stunning (e.g., live handling) and as such has some welfare advantages compared to electrical stunning. Commercial CAS systems most commonly expose conscious birds to increasing concentrations of carbon dioxide (CO_2). CO2 has anaesthetising properties; however above certain concentrations exposure can cause pain (e.g., burning in the nose). During CAS, although painful levels of CO_2 are avoided while the chickens may be conscious, these gases cause a deep breathing (hyperventilation) response, expressed by obvious changes in behaviour (e.g., gasping). It is unknown whether these behavioural changes correspond to sensations reported in humans such as breathlessness and 'air hunger' when inhaling CO_2. Air hunger is reported by humans as a deeply unpleasant experience, consisting of an uncomfortable urge to breathe which is associated with profound anxiety, frustration and fear. We do not know if birds experience these sensations and the consequences, they have on animal welfare. If the gas mixtures used in CAS cause air hunger in chickens, this constitutes a very significant welfare issue, calling into question whether CAS is a humane method of pre-slaughter stunning.

Given birds are capable of flight at high altitudes, and have a unique respiratory system compared to mammals, the susceptibility of birds to air hunger is difficult to predict, and no previous studies have specifically examined the emotional responses of birds to these sensations. Although some earlier studies have demonstrated that chickens avoid CO_2, research has never addressed why, and more importantly if, air hunger plays a part in this avoidance. Simple behavioural tests, such as allowing birds to leave a space containing CO_2, are flawed since CO_2 is an anaesthetic that is likely to slow or impair the bird’s responses. Therefore, more sophisticated behavioural approaches such as those allowing birds to demonstrate if they remember negative emotions associated with exposure to CO_2, and if so, whether these emotions are reflected by birds choosing not to re-enter environments where this occurred.

Tackling the question of whether air hunger is experienced by billions of chickens undergoing CAS is extremely important. The work we propose is novel and answers a fundamental question, which will enable the development of a novel methodology to detect air hunger in birds. If our studies show that air hunger is not an issue relevant to poultry slaughter, CAS will be proven to be a high welfare method and its uptake will be enhanced globally. If air hunger is apparent, the results will drive the development of more humane stunning approaches, ensuring that the current transition away from electrical stunning systems represents genuine welfare improvement. Whatever the findings, the work will provide valuable information to inform future policy globally.

What outputs do you think you will see at the end of this project?

Breathlessness is a potentially major animal welfare issue, and this project will represent the first ever systematic study to identify this perceived sensation in any non-human animal. We anticipate new information in the form of publications with a minimum of one open access scientific peer-reviewed paper published for Experiment 1 (year 1) and a minimum of two scientific peer-reviewed papers from Experiment 2 (year 2 and 3). Conference attendance will maximise dissemination across all relevant scientific and commercial communities nationally and internationally. We will also maximize accessibility

to our work by, where appropriate, publishing associated data on digital repositories or otherwise making it available upon request.

Who or what will benefit from these outputs, and how?

Our overarching goal is to investigate the welfare of chickens at slaughter and killing, and whatever the findings, the results will provide important benefits for animal welfare with the capacity to improve the end-of-life experience for all chickens slaughtered commercially by CAS (billions of birds) and underpin future policy and legislation. Our project offers broad benefits to the academic communities that are concerned with animal welfare, bird biology, and the basic physiology of breathing and how this can be altered by disease. Our completely novel collective approach combines lung and brain physiology and sophisticated behavioural tests to identify air hunger as an emotional state. In addition, the model we create could readily be extended in future to mammals, going well beyond the immediate need to understand whether gas killing in slaughterhouses (e.g., pigs) and laboratories (e.g., rats and mice) is humane (although these very important problems alone justify the effort). The work will directly contribute to the development of an established animal model of air hunger which will enable many physiological studies of neural pathways and experimental treatments applicable to humans and animals.

Whether air hunger is identified as a welfare issue in birds or not, the findings of this project will be directly relevant to academics who form part of advisory committees for Non-Governmental Organisations (NGOs'), government, and veterinary organisations, who are highly influential in guiding national and international policy making and regulatory efforts to improve welfare at slaughter and killing (including Schedule 1 methods of killing for birds under the Animals (Scientific Procedures) Act 1986). Furthermore, findings will inform abattoir, depopulation and slaughterhouse operators with refined welfare indicators for assessment of poultry during stunning and killing. Knowledge transfer will be ensured through publications, conference presentations and direct interaction with relevant bodies such as the Humane Slaughter Association, American Veterinary Medical Association, British Veterinary Association, World's Poultry Science Association, European Food Safety Authority, Department for Environmental, Food and Rural Affairs (DEFRA) and devolved administrations via the Animal Welfare Committee.

How will you look to maximise the outputs of this work?

In line with United Kingdom Research and Innovation (UKRI) funding requirements, the project outcomes will be disseminated through Open Access publications in leading international journals. Conference attendance will maximise dissemination across all relevant scientific communities nationally and internationally. We will work alongside NGOs' as well as government and veterinary organisations who are highly influential in guiding policy and regulatory efforts to improve welfare at slaughter and killing and will hold stakeholder workshops to disseminate our findings. We will also maximise accessibility of our work by publishing associated data on digital repositories. Members of the team have significant previous experience of sharing research findings and promoting regulatory reform, which will maximise the impact of the work undertaken.

Species and numbers of animals expected to be used

- Domestic fowl (Gallus gallus domesticus): 1080

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

There are no alternatives to the use of conscious animals for this work since we aim to establish the welfare implications of exposure to gas mixtures which are relevant to controlled atmosphere stunning. Animal welfare assessment involves assessing behavioural, physiological and psychological elements that contribute to the animal's overall experience (e.g., emotional state) and these cannot be reproduced outside of living organism (in vitro). We will work on female commercial laying hens (e.g., those reared to produce eggs) which prevents issues associated with the rapid growth rate of broilers (e.g., those that are reared for meat) such as lameness due to their legs being unable to support their heavy bodyweights. The rearing of male layer birds to maturity poses unnecessary welfare concerns (e.g. aggression). We expect hens to be representative, given that the fundamental nature of this work to explore the consequences of commercially relevant gas exposure during commercial killing (e.g. controlled atmosphere stunning or killing). There is no evidence to suggest differences in the basic respiratory physiology between broilers and hens (both Gallus Gallus domesticus) nor between sexes or commercial chicken strains. Broiler breeders (individuals that supply the chickens that are commercially raised for meat) are not appropriate, due to required severe feed restriction to rear them post juvenile stages and the associated animal welfare harms (e.g. chronic hunger), as well as confounds with behavioural training (e.g. due to hunger). We may work with adult (laying hens) or juvenile (layer pullets) birds to allow sufficient time for the birds to learn the behavioural tests being used and become used to the handling used and the laboratory environment.

Typically, what will be done to an animal used in your project?

Typically, hens will be socially housed in groups and will be provided with environmental enrichment (e.g., nest boxes, perches, foraging material, etc.). The hens will be allowed to get used to the laboratory and all aspects of the procedures including handling, gentle restraint, social isolation and novelty of the testing apparatus. Hens in all procedures outlined in this licence will be exposed to gas mixtures across multiple sessions (maximum 6 sessions in total; maximum of 3 exposures per day). These gas mixtures are not considered to cause pain based on previous work (birds will be extensively monitored throughout), and individual sessions will not last longer than 5 minutes. Behavioural and physiological responses will be measured and monitored. Hens utilised in experiment 1 (protocol 1) will be humanely killed (Schedule 1) upon completion of the experiment and tissues sampled for molecular biology (e.g., to determine physiological changes in the tissues). For hens undergoing experiment 2 only (protocols 2-5), attempts will be made to rehome them in accordance with ASPA Section 17A section (3) and (4). If rehoming is unsuccessful hens will be humanely killed (Schedule 1).

What are the expected impacts and/or adverse effects for the animals during your project?

The hens will experience gentle restraint and exposure to gas mixtures. These gas mixtures are not considered to cause pain, but they may experience some mild discomfort associated with hyperventilation (with its quantification and nature being the overarching aim of this project). All birds will be continuously monitored both behaviourally and physiologically to determine the full welfare consequences of increases in carbon dioxide

(i.e. known as hypercapnia) and decreases in oxygen concentrations (i.e. known as hypoxia) in gas mixtures they are exposed to. However, gas exposure will be brief in nature to replicate approximate exposure times in controlled atmosphere stunning.

Additionally, the exposure to non-painful gas mixtures for short periods acts as a refinement, whereby we limit the impact of the mixtures on the animals physically or psychologically. We do not anticipate any long-lasting effects of the gases and for the experiences to be mild both for individual exposure sessions, but also when considering repeated exposures (maximum 6 sessions).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild: 95%
Moderate: 5%

What will happen to animals at the end of this project?

- Killed
- Rehomed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

There are no alternatives to the use of conscious animals for this work since we aim to establish the welfare implications of exposure to relevant gas mixtures. Animal welfare assessment involves assessing physiological and psychological elements that contribute to the animal's conscious experience and these cannot be reproduced in vitro.

Which non-animal alternatives did you consider for use in this project?

There are no non-animal alternatives available.

Why were they not suitable?

Animal welfare assessment involves accessing physiological and psychological elements that contribute to the conscious experience of animals and these cannot be reproduced in vitro.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot

studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have adopted good experimental design practices and have designed our studies carefully to ensure the use of a minimum number of birds required to observe meaningful effects for reliable data with the inclusion of appropriate controls. The number of hens has been calculated using the appropriate statistical methods for each experiment using behavioural data from previous relevant studies. Birds will be randomly allocated to their experimental groups in accordance with robust experimental design practices and where possible, the observer blinded to the experimental treatment (for both gas concentrations and drug intervention).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Extensive consideration has been given to the proposed experimental design along with the methodologies required to fulfil the aims of the project whilst minimising the number of birds required. Sample sizes have been determined based on previous work that has been carried out to determine the minimum number of birds required to observe meaningful effects for any work conducted under this licence. The researchers involved in this project have extensive expertise in designing such studies and have consulted and an independent statistician to ensure robust experimental design.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will use a stepwise approach throughout this project with Protocol 1 and 2 forming the basis (pilot work) for subsequent work outlined in Protocols 3, 4 and 5. Further, we will adopt experimental designs (e.g., within subjects) to reduce variability between individual birds and so reduce number of animals required.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

This work involves determining the welfare impacts to poultry of gases used during commercial slaughter and therefore to achieve the aims of this project, we must use the target species concerned. Chickens can be split into two distinct types: meat birds (broilers) and egg producing birds (laying hens). We will work with a commercial strain of laying hens to prevent issues associated with the rapid growth rate of broilers, thus allowing utilisation of behavioural tests that require extensive training.

Broiler breeders are not appropriate, due to required severe feed restriction needed to

allow birds to reach maturity and the issues this would cause with training for behavioural tests. Commercial laying hens are all females, in order to produce eggs, with males of these lines killed as newly hatched chicks. The rearing of male layer birds to maturity poses unnecessary welfare concerns (e.g. aggression). We expect hens to be representative, given that the fundamental nature of this work to explore the consequences of commercially relevant gas exposure during commercial killing (e.g. controlled atmosphere stunning or killing). There is no evidence to suggest differences in the basic respiratory physiology between broilers and hens (both Gallus Gallus domesticus) nor between sexes or commercial chicken strains.

Under this license, birds could experience some potential mild and transient discomfort associated with gentle restraint, exposure to gases used for commercial slaughter (which in itself remains the focus of the research) and with the administration of anti-anxiety medication by the most refined injection route for the compound. We cannot utilise oral routes as it would likely require additional social isolation to ensure correct dosage and as hens socially feed, they are unlikely to voluntary feed when isolated from their pen mates. Additionally oral feeding would not be optimal for several reasons, including uncertainty of individual dosing if supplied in the feed of group housed animals, the possibility that animals may need to be temporally food restricted prior to testing, and the requirement of larger doses due to metabolism by the liver when using the oral route, and why we have opted to only consider injectable routes. We do not anticipate any adverse effects as all birds will be extensively acclimatised to all aspects of the experiment, and gas exposure within an individual session is expected to be brief (<2 minutes), non-fatal, and below concentrations capable of eliciting pain (e.g. behavioural aversion in chickens has been demonstrated by withdrawal from feed in CO_2 concentrations >25% and nasal mucosa nociceptors activated by CO_2 concentrations >40%) or loss of consciousness (>30% CO_2).

The exposure time has been limited to <2 minutes to mimic the gradual exposure to the gases relevant controlled atmosphere stunning systems for poultry and the maximum time chickens may be exposed to these gases prior to losing consciousness if stunning/killing parameters were followed. Additionally, birds will not be subjected to more than 10 repeated exposure sessions (maximum of 10 sessions in total; maximum of 3 exposures per day). This schedule has been designed to ensure full respiratory recovery between exposures of non-aversive concentrations of gas and limit the time in the cradle (and associated social isolation) to 30 minutes which has been previously documented as being well tolerated in laying hens based on previous studies by the project team as well as in the scientific literature, which report up to 1hr. Additionally, only birds fully habituated to the apparatus will be exposed to gas treatments and being housed in the cradle for the maximal time of 30 minutes.

Therefore, there is a low risk of adverse effects (including cumulative effects), with a number of refinements in place. All birds will be closely monitored throughout handling/restraint, drug administration, gas exposure as well as during training and habituation (e.g., acclimatisation to the study and environment). Habituation periods for the experimental apparatus will be extended (if necessary) to ensure birds are comfortable in the apparatus and therefore any findings identified are related to our experimental treatments only.
Birds will be housed in stable social groups to promote species typical behaviours and will be housed under conditions that will provide better welfare outcomes than those used across the commercial sector.

Why can't you use animals that are less sentient?

The work involves determining the welfare impacts of chickens exposed to gases relevant to those used during commercial slaughter and this requires us to assess the behavioural, physiological and psychological effects in the relevant species. As a result, we cannot conduct the work in other animal species or in anaesthetised birds. Assessing welfare involves accessing the physiological and psychological elements that contribute to the animal's experience and these cannot be reproduced using non-animal alternatives or less sentient animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Birds will be socially housed in stable groups and will be housed under enriched conditions for the duration of the study. Birds will be extensively acclimatised to all aspects of the laboratory including handling and restraint using low stress methods to minimise the impact of procedures outlined in this licence that require handling. Any treatments applied will be administered using the most efficient and non-invasive means possible. All birds will be closely monitored by technical staff and researchers using established scoring systems. The birds will be housed above, or at, standards in accordance with the Code of practice for the housing and care of animals bred, supplied or used for scientific purposes (2014).

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Alongside the guidelines listed below, I will also adhere to local AWERB standards for research animals, and where appropriate, support the development of new local standards for refinements discovered during the project licence.

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
NC3R's and Procedures with Care
We also follow the advice provided by the local Animal Welfare Ethical Review Board, Named Information Officer, Named Animal Care Welfare Officer, Named Training Competency Officer and Veterinary team.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Officer (NIO), Named Animal Care Welfare Officer (NACWO), Named Training Competency Officer (NTCO) and Veterinary team regularly inform and disseminate improvements and recent studies involving reduction, replacement and refinement to the researchers across our institution. We routinely use external resources, including collaborators, peers, scientific conferences, and laboratory animal and animal welfare bodies. During the 1, 3 and 5-year review of the project licence, we will update on the implementation or consideration of the 3Rs that have occurred during the previous period, alongside a review of the linked training plan, score sheets etc., in collaboration with the NACWO, NIO, NTCO and veterinary team with a particular focus on refinements.

We have assembled extensive and international animal welfare expertise in the project team, and therefore we are fully aware of the ethical and legal considerations relevant to the use of animals for scientific purpose and are actively engaged in implementing the principle of the 3Rs. Importantly, our institution is an internationally recognised university where adherence to the NC3Rs principles is a primary focus of all animal care staff and researchers. Furthermore, the applicant is a member of established United Kingdom Research and Innovation (UKRI) Grant Review Panels as well as being a member and chair of multiple ethics panels. The project team attends regular talks and discussions about animal welfare and the 3Rs through research presentations by staff and students at research meetings within our institution. Many of those meetings focus on developing new strategies to assess adverse impacts and promote positive welfare refinements in research animals, e.g., handling procedures to reduce distress.

45. Mechanism and functional role of calcium signals

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Calcium, Reproduction, Metabolism, Cancer, Heart

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 the project is to understand the role of calcium signals, that is information mediated by changes in the concentration of calcium in the cell, as regulators of a range of cellular and bodily processes related to health and disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Changes in intracellular calcium constitute a central signalling mechanism in the cell and organism. Abnormalities in calcium signalling mechanisms underlie many different types of normal physiological processes, but also processes linked to disease, in humans. In this project I will study the role of calcium signals in processes linked to diseases such as infertility, heart attacks, cancer, and diabetes.

My findings will both lead to a better understanding of these diseases and could lead to new diagnoses and treatments for them.

What outputs do you think you will see at the end of this project?

Outputs will include new information and publications. It could ultimately pave the way for the development of new drugs to treat human conditions such as infertility, diabetes, heart disease, cancer, and disorders of the immune system.

Who or what will benefit from these outputs, and how?

Other academics will benefit from the new information provided. In the long run, the findings of this project could lead to the development of new types of diagnostic methods and treatments for disease. A key focus of this project is on better understanding the role of calcium signals, and the mechanisms of action of calcium signalling proteins we are studying such as PLCzeta and the two-pore channels (TPCs) in disease states such as infertility, cancer, heart disease, diabetes, and disorders of the immune system. Thus, in the long run, a benefit could be the development of new ways to diagnose and treat such disorders.

How will you look to maximise the outputs of this work?

We will look to maximise the outputs of this work through publications and presentations at conferences.

Species and numbers of animals expected to be used

- Mice: 9000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The animals used in the project are adult mice. Mice are one of the key mammalian organisms for studying pathophysiological processes. Mice have a short gestation period, are economical to keep, and are very well established as a model for studying the role of genes in the body. We will not study mice at earlier stages of development as our interest is in the role of calcium signals as regulators of pathophysiological processes in the adult. However, we will study sperm and egg biology in gametes derived from adult mice.

Typically, what will be done to an animal used in your project?

Genetically altered mice will be bred which is not expected to have any adverse effects as the gene knockouts we are studying have no record of anything greater than a mild phenotype, including in older mice. They will then be maintained up to a maximum of 15 months of age when they will be humanely killed to allow us to harvest cells, tissues, and organs for use in in vitro experiments in the laboratory.

Some female mice will undergo the administration of agents, typically gonadotrophin, hormones via the intraperitoneal or subcutaneous route to induce superovulation (the production of exceptional numbers of ova at one time). Mice may be mated with males to produce in vivo embryos. The female mice will be humanely killed no later than 24 hours post the last administration and unfertilised ova or embryos harvested.

What are the expected impacts and/or adverse effects for the animals during your project?

The mice have a mild phenotype and will have no adverse effects as no such effects have been detected in the gene knockouts we are studying. The injection to induce superovulation is also a mild procedure that will cause only a mild and transient discomfort to the animal.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

85% subthreshold and 15% mild.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

For studying many pathophysiological processes, whole animals are necessary as studies of immortalised cells in culture differ in many important ways compared to primary cells taken from a whole animal. Whole animals are also important for studying how different cells, tissues and organs interact and influence each other.

We will study the effects of such influences in processes in different cell types, tissues, and organs, through our ex vivo analysis.

Which non-animal alternatives did you consider for use in this project?

We already extensively use immortalised animal and human cells for our studies which are thus an alternative to use of whole animals.

Why were they not suitable?

It is impossible to fully recreate the environment of the whole organism and the complexity of its cells and organs with immortalised cell culture systems. Some cell types e.g. sperm and eggs, have no equivalent in immortalised cells. All primary cell types differ from immortalised cells in a variety of ways, so it is important to have an ex vivo source of e.g. pancreatic acinar cells, hepatocytes, cardiac myocytes, as well as organs such a whole heart, brain, etc.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 the data from previous experiments to assist us in estimating the numbers we require for each experiment. Statisticians at our establishment have assisted us in previous projects and we will continue to consult with them. Typically, we require groups of n=3 for our experiments. We have also used our previous home office returns to assist in estimating numbers for our breeding programme.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

All strains are on a 100% C57BL/6 background, the standard for numerous studies, to minimise variability. Homozygous PLCz1 KO and KI mice will be produced by breeding heterozygote animals, TPC1 KO, TPC2 KO, TPC1/2 double KO, CD38 KO, and other related KO and KI strains will be bred as homozygous colonies. We will aim to use each animal as a source of multiple primary cell types, tissues, and organs, rather than for just as a source for a single cell type/tissue/organ, to minimise the numbers of animals used in the various aspects 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?

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. Where we harvest cells, tissues, and organs post-mortem we will offer those not needed for our experiments to others. We will also store harvested tissues, organs, and cells for future experiments rather than using more animals.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will be using genetically altered mice in this project that have a mutation and/or deletion in calcium signalling pathways. In particular we have a special interest in PLCz1 KO and KI mice, and TPC1 KO, TPC2 KO, TPC1/2 double KO mice, and CD38 KO mice,

and related KO and KI mice. These mice strains will be bred using conventional methods. Our phenotypes are not harmful, and, in most cases, we can breed homozygous animals together to avoid both excess animals of a genotype that are unsuitable for our experiments and remove the need for tissue biopsy to confirm the genotype of the animal. Animals will be humanely killed at various ages, up to a maximum of 15 months of age, and cells, tissues and organs harvested to assess the role of calcium signals in a variety of pathophysiological processes in vitro. Where we wish to study the calcium signalling pathways in immature life stages, we will take female mice and inject them with agents such as gonadotropin to produce exceptional numbers of ova at one time – known as superovulation. Whilst this does mean that female mice will be subjected to up to two injections, the large number of ova available to us means that we need to use fewer animals. Where fertilized ova are needed the females will be mated.

Why can’t you use animals that are less sentient?

In the past, we have used non-mammalian species such as sea urchins and zebrafish to study the mechanisms of action and pathophysiological roles of PLCz1, TPCs, and CD38. However, our focus is now on aspects of pathophysiology that are specific to mammals, hence the focus on mice, as an important mammalian model of human health and disease.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The procedures we are using in this project (breeding and superovulation) are both well refined and therefore we do not expect to need to refine them further. Animals will be monitored daily by staff in the Oxford University Biomedical Sciences building and this will further help minimise any potential harms to the animals.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the LASA, ARRIVE and PREPARE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will read the relevant literature and websites. We will be attending 3R events and welfare meetings held at the establishment and we will have signed up to the NC3R’s website. We will also liaise with the establishment's Named Information Officer to ensure that we keep abreast of any new advice.

46. Mechanisms of airways infection and inflammation

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Asthma, Infection, Immunology, Epigenetics, Obesity

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?

Investigation of the causes of inflammation in lung diseases including bacteria, allergic inflammation, lung scarring and long-term changes in the control of genes (‘epigenetics’).

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Asthma is the world’s commonest long-term lung disease, affecting >350 million people. Asthma seriously affects quality of life: 1/3 of sufferers find asthma interferes with leisure activities and 1/4 with their ability to work or study. Asthma carries a massive economic burden, with 4.1 million lost working days in UK per year, despite £1 billion of NHS spending. Asthma remains a terrifying and potentially fatal condition, claiming 3 lives each day in UK. These deaths, and most symptoms are caused by ‘exacerbations’ also called ‘asthma attacks’. These are usually caused by viral or bacterial infections which activate immune cells of the airways to cause inflammation. However, the mechanisms underlying

this inflammation are poorly understood and are considered the top priority for European asthma researchers to address. Fibrotic lung disease causes progressive scarring of the lungs leading to disability and death. It can occur without clear precipitants and may also occur as a consequence of previous viral infection, or potentially be related to the emerging industry of vaping. It is considered to be due to a failure to control the appropriate responses to tissue damage in the lungs. The causes of different forms of asthma, including how obesity and epigenetics contribute to asthma, are still unknown.

Our goal is to address these problems by asking several questions. How do bacteria become established as infections in the airways of people with asthma? What effects do they have on inflammation in the lungs? How do bacteria in the airways make us susceptible to viruses? How do particular antibiotics reduce inflammation in airways disease? How can we reduce inflammation and scarring of the lungs? How can obesity trigger the immune changes in asthma? How do nerves interact with immune cells to contribute to airway inflammation? And how do particular epigenetic changes which we identify in our human studies cause features of asthma?

What outputs do you think you will see at the end of this project?

This project, as part of a larger programme of work including in vitro and human studies, is designed to address an important clinical question, arising from the needs of patients in clinic. We anticipate the following outputs:-

New knowledge on immune mechanisms in asthma. This will include better understanding of the interaction between bacteria, viruses and inflammation in asthma; how epigenetic changes affect asthma; and how obesity can lead to development of asthma.

Scientific publications. We anticipate our research programme will lead to peer reviewed, widely disseminated publications allowing researchers to benefit from the insights we generate.

Development of innovative **new scientific model**s of persistent airways infection with pathogens. Specifically there are currently no effective experimental protocols in animals to study the effects in the lung of the most common long term respiratory pathogen – *Haemophilus influenzae* – and we anticipate we will be able to develop protocols which work in mice and can be used by other researchers globally.

Who or what will benefit from these outputs, and how? Patients with airways diseases?

There is a clear unmet clinical need to reduce asthma exacerbations. We anticipate our research will help support development of alternative approaches to treating severe asthma such as identifying alternative medications which have anti-inflammatory properties but are not essential antibiotics whose overuse risks development of drug resistance and side effects. Studies on the immune consequences of weight loss may support parallel clinical trial data we are generating in human volunteers to justify the rationale use of these medications with severe asthma and obesity. In the much longer-term identification of important epigenetic mechanisms could identify targets for potential curative treatments for asthma.

Timescale: Data on rationale use of existing obesity drugs: expected clinical translation within 5 years. Data on potential novel macrolide antibiotics: expected clinical translation 10 years.

The UK economy

Evidence-based use of long-term azithromycin is likely to be a highly cost-effective treatment by reducing some of the 4.1 million working days lost in UK annually to asthma. By potentially obviating the need for alternative, costly biologic therapies, or averting hospital admissions this will be likely to impact on the £1 billion annual cost of asthma to the NHS. The pathway to impact will be through knowledge dissemination amongst colleagues leading other UK severe asthma services.

Timescale to clinical translation: <5 years.

Any potential novel drug target could be of significant economic value to the UK and global economy. The current class of 'biologic' injections in asthma constitute a global market worth $23bn/year.

Timescale to clinical translation: 10 years.

Other researchers

Successfully developing and validating a mouse model of long-term *Haemophilus influenzae* infection would be of benefit to researchers globally who currently do not have any adequate mouse models for this infection.

Timescale to others using this model: <3 years.

How will you look to maximise the outputs of this work?

Research findings will be disseminated as widely as possible via peer reviewed, open access journals. Open access publication is a requirement of our funding, as is deposition of all sequencing data in publicly-accessible repositories.

We will also disseminate scientific information at conferences including the British Thoracic Society, European Respiratory Society and the Society for Mucosal Immunology.

Mouse models will be available via publication.

We will also remain active in public engagement with standard press releases and continuing engagement with TV, radio and written communication channels.
We also interact directly with patients with asthma, both locally (through our Patient Participant and Involvement (PPI) group and through our PPI events) as well as nationally through the Asthma+Lung UK network supported by their outreach and PPI team.

Species and numbers of animals expected to be used

- Mice: 12200

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The adult mouse is the most reliable and well-validated model of innate and adaptive immune responses and is the most widely-used species for studying mammalian immunology. For these reasons there are many complex biological tools available to work with this species, such as antibodies, and it is superior to the current Chinchilla model for persistent infection with the bacterium *Haemophilus influenzae*. It is not possible to model haemophilus infection in lower species as this bacterium is uniquely adapted to mammalian tissues, and there are a range of highly relevant mouse genetically modified strains available, which are essential to this project. The work builds on previous work over the past 5 years in which we have optimised our model systems in the adult mouse.

Moreover adult mice are used as they are large enough for us to undertake lung function measurements, necessary for some experiments.

Typically, what will be done to an animal used in your project?

Genetically altered mice will be bred. The phenotypes produced are not expected to cause any harms that are more than mild and transient.

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.

Some animals may receive medications or substances in food or drinking water or by oral gavage. Some diets or substances may result in weight loss due to unpalatability. Animals will be placed onto normal diet should they lose >10% and <15% of their body weight.

Typically, animals will also receive a lung infection with a bacterium or virus, administered under brief inhalation anaesthesia. For up to 72 hours they will experience mild or moderate transient ill health (displayed by ruffled hair and/or slightly reduced movement) and up to 15% transient loss of weight (or up to 20% with specific bacteria or viruses, as enumerated in Table 1, where rapid recovery is expected, and where weight loss >15% will only be tolerated for up to 48 h).

Where lung fibrosis is being studied, typically animals will receive administration of a substance intranasally or intratracheally under brief inhalation anaesthesia. They will experience mild or moderate transient ill health (displayed by ruffled hair and/or slightly reduced movement) and up to 20% transient loss of weight (where weight loss >15% will only be tolerated for up to 48 h), peaking at 72 hours and resolving by day 7.

Where the effects of allergic airways inflammation (asthma) are being studied mice will typically receive repeated doses of allergens by aerosol (exposed whilst alert and free running in a cage) or intranasally (up to 7 doses per week under brief inhalational anaesthesia). This is usually well tolerated causing only mild airway inflammation with no symptoms. This is in line with recent, more refined models of allergic airway inflammation, which avoid use of high-dose intraperitoneal sensitisations and instead use chronic, repeated low dose administration of allergens to better recapitulate the true pathology of human allergic asthma.

In experiments investigating obesity, animals will undergo changes in diet which are not expected to cause distress but may result in obesity.

Animals will experience mild and transient discomfort from blood sampling.

The final procedures, including measurement of lung function, will be undertaken under non-recovery anaesthesia where the animals will only be aware of the anaesthetic being administered and will experience no pain.

What are the expected impacts and/or adverse effects for the animals during your project?

Mice receiving an injection into their vein, skin, or abdomen, or blood sampling will experience mild transient pain lasting a few seconds, just like people. If receiving a vein injection or blood sampling this will also involve restrained for up to 1 minute during the procedure.

Some mice requiring administration for prolonged periods, will have minor surgery to implant a device under the skin that can release a medicine slowly, to avoid the need for repeated injections. They are expected to recover quickly and will be given painkillers and post-operative care just like people recovering in hospital.

Most animals will receive a substance under inhalational anaesthesia on at least one occasion. They receive this in a cage and may find the smell unpleasant. The anaesthetic administration takes from between 1 and 5 minutes typically, and effects wear off within a few minutes, with no lasting effects.

Infections with most bacteria and viruses will lead to symptoms for up to 72 hours during which they will experience mild or moderate transient ill health including fever, lethargy (displayed by ruffled hair and/or slightly reduced movement) and up to 15% transient loss of weight lasting up to 5 days, with full recovery of weight expected by 10 days. Certain specific infections can cause more rapid weight loss and recovery, in which case mice may experience transient weight loss of between 15-20%, which would be tolerated only for up to 48 hours.

Administration of substances causing lung fibrosis will lead to transient ill health (displayed by ruffled hair and/or slightly reduced movement) and transient loss of up to 15% of body weight. At certain doses this can cause more significant weight loss and recovery, in which case mice may experience transient weight loss of between 15-20%, which would be tolerated only for up to 48 hours. Some animals participating in longer experiments may experience laboured breathing or disordered breathing rhythm after 2-3 weeks, but which would not be expected to prevent the animal’s ability to eat and drink, as might be experienced by a patient with many long term lung conditions.

Where allergic airways inflammation is studied most mice will experience no symptoms, and changes are detectable only using lung function measurements or immunological tests at the end of the experiment.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Subthreshold: 10% Mild: 31% of mice Moderate: 49% of mice

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Fundamentally the questions we are asking relate to the behaviour of a fully intact immune system, including many different cell types which interact with each other, and across the whole respiratory tract. It is therefore not possible to address the importance of specific factors *in vitro*, even though we will maximise the use of *in vitro* assays to confirm key findings. Furthermore, we are also addressing physiological questions, which require analyses of changes in the whole airway system, comprising a number of different tissue such as epithelium, smooth muscle and glandular tissue, in addition to immune tissue. This complexity is not yet able to be replicated *in vitro*.

Which non-animal alternatives did you consider for use in this project?

We will nonetheless model certain aspects of the disease *in vitro*, and are funded to do such work in parallel. If we have indications from these cell culture models which could lead to replacement of some of the planned *in vivo* work we would take advantage of this. Specifically we use human cell lines *in vitro* and also human primary airway cells which we can grow in submerged culture or at air liquid interphase. Furthermore we are now undertaking *in vitro* analyses using human induced pluripotent stem cells (cells grown in the lab which start from blood samples or skin biopsies in volunteers and can then be turned artificially into other cell types), which over time may enable more aspects of asthma biology to be studied without the need for animals.

We are also conducting parallel human studies using bronchoscopies (inspection of the airways using a flexible camera under light sedation). The experiments planned are only those where we cannot achieve the same objectives by analyses of human subjects. These data, including analysis of human innate T cell responses can be used to plan better experiments in the mouse model, which can then be used to address questions most relevant to the clinical setting. Human volunteers undergo bronchoscopies under sedation to provide samples of human airway tissue for techniques such as single cell sequencing and microbiological studies, and in some cases these volunteers also take clinical treatments such as antibiotics and weight loss treatments.

Why were they not suitable?

In vitro studies with cell lines, human stem cells or human primary cells from bronchoscopies currently allow us to study only one, or at most 2-3 cell types at a time in this manner, so for most questions these are not able to recapitulate the complex inflammatory and physiological networks which interplay in the intact animal.

Bronchoscopy studies are very important as they directly study real human disease. However many experiments are impossible in humans, for instance it is not ethical for us to deliberately infect our human asthma volunteers with bacteria or viruses, or to induce asthma or lung fibrosis in healthy volunteers which would cause lasting damage.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Numbers are based on our prior experience with experiments of this design and these specific models. 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 5-8 to achieve the quality of results we need, or 8-25 for some key lung function measurements. Many experiments are exploratory and descriptive or needed for optimisation and will be performed once in a fully powered experiment. Key experiments will be repeated once (or twice if low consistency between replicates) to confirm reproducibility, with data presented as combined from experiments. 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?

We are breeding only animals numbers appropriate for the required experiments. Wherever possible multiple outcomes will be measured simultaneously to minimise animal numbers.

To minimise variation I will using blocking to stratify the key nuisance variables between replicates which are variation in the experimental inoculum administered and age of mice.

To reduce bias we will use blinded, random allocation (via eda.nc3rs.org.uk) within cages for administration of ligands, antibodies, azithromycin and steroids.
We will be conducting our experiments so that we 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. 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. Where unavoidable excess animals become available from breeding protocols they are shared with other researchers where these may be of use.

Where a strain is not required for a prolonged period it will be cryopreserved and breeding ceased.

Where possible tissues from a single animal will be used to provide multiple readouts (for instance histopathological, microbiological, measures of gene expression, and cellular immunology all from a single animal). Where possible tissues from other organs (typically bone marrow or spleens) will be shared with other researchers within the group, or neighbouring laboratory group.

Any new protocol will be tested in a small pilot using just 3 mice per experimental group, to optimise doses or timepoints to ensure most efficient use of animals in definitive experiments.

Both sexes will be used for experiments, which ensures generalisability of findings and minimises inefficient breeding.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mouse strains: We will use strains of mice which have alterations in their innate immune response which make them susceptible to infection or to inflammation. They do not show a phenotype prior to challenge with infection or inflammatory stimuli. Others have modifications in their T cells which allow us to better identify certain types of T cell, or to deplete those specific cells when they are given gene activating substances such as Tamoxifen.

General principles: Models will include induction of bacterial and viral infection by intranasal or intratracheal inoculation; induction of allergic airways inflammation using intraperitoneal/intranasal sensitisation followed by repeated intranasal or aerosol challenge with allergen; induction of inflammation using oral exposure of a genetically inducible mouse strain; induction of sterile lung injury using intratracheal or intranasal bleomycin.

The principles we use to select models are that we chose models which have either been widely published and refined by others, or have been refined in our own work, and which are selected to provide sufficient immunological changes to answer the questions being addressed but with the minimal concomitant distress to the animals. This is particularly in terms of minimising weight loss, which is frequently a useful overall marker of the extent of ill health experienced by an animal. We have also selected models which most realistically simulate aspects of asthma, based on a review of current literature – several widely used older models have been criticised in recent years for being unphysiological and unrepresentative of the changes which occur in real human disease.

Haemophilus infection model: This is a relatively new model that we have developed and refined during our previous project. *Haemophilus influenzae* gives us a persistent bacterial injection using a genetically altered susceptible mouse strain. The model is simple, reproducible and replicates the human situation without any toxic side effects or the need for repeated infections. This model only causes transient illness which resolves in 10 days despite the ongoing infection in the airways.

Other infections: we will infect mice intranasally with other relevant human pathogens which cause airways infection; *Moraxella Catarrhalis*, *Pseudomonas Aeruginosa, Streptococcus Pneumoniae, Staphylococcus aureus*, Human Rhinovirus, Respiratory Syncytial Virus (RSV) and Influenza A. Extent of weight loss: most experiments with these protocols induce <10% weight loss, but in some cases more significant weight loss is necessary to recapitulate the actual immunological events occurring in human lung infections. Specifically we have shown that the model which requires expansion of innate T cells using intranasal Salmonella infection, only generates significant expansion of the cell population with an infectious inoculum causing transient 15-20% weight loss. Likewise infections using influenza virus or bleomycin require transient 15-20% weight loss to induce the immunological effects we are studying.

Duration of protocols: acute infection models typically last just 48 hours, whilst chronic infections will typically last for 7 days, though symptoms tend to resolve over this period.

Allergic airways inflammation: we will induce airways inflammation using a common aeroallergen, typically house dust mite allergen, which is of low toxicity and simulates what many of us are exposed to daily in our homes. For long term exposures (more than 7 doses), to avoid the stress related to recurrent anaesthesias, where pilot data are supportive, the mice may be exposed by aerosol into their cages and this allows them to be conscious, free running and relaxed. For shorter term exposures up to one week inhalation anaesthesia is used because this allows accurate dosing with the minimum quantity of allergen. Such recurrent, low dose exposure regimes are preferable scientifically to older, short term, high dose exposure models which do not induce changes typical of real human asthma or realistic environmental exposures. Our protocols last several weeks because allergic airways disease typically develops over several years in humans: to recapitulate key aspects of this in a mouse is not possible in less than a few weeks as the immune response takes time to develop.

Expansion of innate cell subsets: expansion of innate cell subsets occurs with a transient infection model using intranasal Salmonella infection which has been developed and refined and published by my former research group and in my own research group: we know the optimal dose regime to produce the necessary immunological effect and that full recovery of weight occurs within 5 days.

Induction of lung fibrosis: Our model of choice for lung fibrosis is the administration of bleomycin, a type of anticancer therapy. This model is widely used and is reliable, reproducible, predictable and causes only transient weight loss but still provides us with the lung fibrosis that we would see in the human patient.

Administration of bleomycin can be done under general anaesthesia via rapid instillation removing the need for any surgical intervention or via the intra-oral cavity which only involves a gentle extension of the neck to achieve. Weight loss can be between 15 and 20% with this model but it is required to induce the immunological effects we are studying. The typical duration of the experiment following induction is just 48 hours but for chronic studies this may be up to 10 days.

Why can’t you use animals that are less sentient?

The adult mouse is the most reliable and well validated model of innate and adaptive immune responses and is also considered the mammalian model of lowest sentience. Non mammalian species have a very different immunological response which provides limited insight into these highly-disease relevant questions we are asking, and some typical experimental species (fruit flies and nematodes and zebra fish) also lack lungs.

More juvenile mice cannot be used for several reasons: 1) allergic airways inflammation and persistent bacterial infections take time to develop as the chronic immune response is slow (weeks) 2) younger animals are more susceptible to infections and likely to suffer greater sickness / more severe experience of the infection 3) lung physiology is challenging to measure in the adult mouse, but is not possible in much younger animals as the trachea is too small to surgically cannulate.
Terminal anaesthesia is used for measurement of lung function, but it is not possible at earlier stages in the protocols because it is only practicable for minutes to hours, but immune responses takes days/weeks to develop.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

For infection experiments the choice of inoculum is based on either our in house experience and refinements or on literature review of studies which have already refined the infection models on WT mice to optimize endpoints and readouts, whilst minimizing suffering to the animals and ensuring sub- lethal doses. The animals will be closely monitored to detect disease progression and ensure animals are euthanized at the appropriate intervention point.

Route of infection will be intranasal where possible as this is the most refined route. As an alternative the intratracheal route may be used if this is necessary to deliver a very precise inoculum to the lower airways and because it may reduce the extent of upper airway pathology (such as otitis media) which may be a scientifically irrelevant consequence of the inoculation. Only a non-surgical approach will be used for intratracheal infection.

Animals will be checked regularly after infection or fibrosis procedures and pain/distress will be assessed using a defined score sheet developed with the welfare officer. Analgesia can be provided after any protocol step, including irradiation, infection or fibrosis procedures if evidence of pain or distress is observed. Such analgesia will be given whenever possible, and under NVS advice.

To minimise the extent of inflammation, necrosis and potential pain and distress associated with procedures that require adjuvants we will not use adjuvants that are known to cause skin irritation which may ulcerate but use a less inflammatory product.

Where possible mouse adapted strains of influenza (eg X31 strain) will be used as they are of lower virulence in the mouse and therefore better tolerated.

Infected mice will be monitored daily in the first 3 days post infection, or longer and up to twice daily if required for specific protocols or during periods of significant weight loss, according to carefully specified monitoring protocols, and then three times per week until the end of the experiment.

Monitoring will be for any sign of ill health, such as: appearance (hunched) and body conditions (dehydrated), ruffled hair, breathing rhythm, activity (mobility) and/or weight loss. Should any symptom persist or worsen, escalating monitoring (daily to twice daily or even 2 hourly) will be in place. Mice will be humanely killed if their clinical symptoms do not resolve as indicated in the humane endpoints.

Where anaesthesia or analgesia is required, this will be induced using agents and routes appropriate for the species and nature and duration of the procedure. Animals will only receive their next inhalational anaesthetic if they have fully returned to normal.

Blood draws will be kept within approved limits – a maximum of 10% total blood volume in 24h and 15% of the total blood volume in any 28d period.

Aseptic techniques will be used if a terminal procedure should be prolonged beyond 60 mins, although this is not anticipated being necessary.

To improve welfare, during periods of weight loss due to infection or inflammation mice will be provided with moist palatable food at cage floor level to arrest the decline in body weight.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will will follow best practice guidance from Laboratory Animal Science Association (LASA), Animal Research: Reporting of In Vivo Experiments (ARRIVE) and Planning Research and Experimental Procedures on Animals: Recommendations for Excellence (PREPARE) as well as from the NC3R’s.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will ensure we are appraised of advances in the 3Rs through discussion with Vets and Named Information Officer during the conduct of the study; through literature review; through receiving the periodic updates circulated within the establishment's biomedical services user groups and through attendance at internal 3Rs meetings. We will pay attention to best practice resources available at www.nc3rs.org.uk and https://science.rspca.org.uk and implicate recommended refinements as we become aware of them.

47. Movements and behaviour of fish in the natural environment

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

Fish behaviour, Shark conservation, Climate change, Telemetry

Animal types	Life stages
Isurus oxyrinchus	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 study the movement and behaviour patterns of fish in the natural environment to determine relationships between habitat preferences and environmental variations. Processes to be studied include foraging movements, feeding behaviours, habitat selection, migratory pathways, and how these may alter with natural and human-driven environmental changes.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 research will contribute to the fundamental and strategic understanding of the consequences of ocean warming, deoxygenation and fishing exploitation which together have the potential for accelerating declines of already threatened species of apex

predators. Tens of millions of individual sharks are caught each year by high-seas fisheries with significant reductions in catch rates documented for many species which can have unpredictable consequences for ecosystem stability and affect biogeochemical cycling.

There is a critical need for improved understanding of shark ecology around climate-driven expansions of Oxygen Minimum Zones (OMZs) that takes into account future climate change and fishing effects. A key issue is that if OMZs continue expanding in the coming decades as predicted, horizontal and vertical movements of oceanic sharks may be increasingly restricted to the narrow, warm, oxygenated surface layers above OMZs, potentially reducing habitat extent and increasing their susceptibility to capture by fisheries that may further reduce populations. A major unanswered question concerns how the effects of both expanding OMZs and fishing exploitation will shift shark behaviour, habitat use and distributions, and the consequences of this for ocean ecosystem structure and functioning.

What outputs do you think you will see at the end of this project?

The new research addresses directly the recent action stated in the 600-page International Union for Conservation of Nature (IUCN) Report on “Ocean Deoxygenation: Everyone’s Problem” (IUCN 2019) that calls for new data on the responses of fish to ocean changes - specifically ocean deoxygenation - and how these will alter habitat use and vulnerability to capture by industrialised fisheries. Specific outputs will include new data that will be available to publicly available oceanographic databases, the filing of new patent(s) on the sensor tagging technology developed to measure fish responses to deoxygenation, open-access scientific publications, presentations at scientific conferences, and provision of data and reports for public policy. The benefit of the research will be to provide basic scientific information capable of supporting decisions about how to manage and conserve threatened shark populations in the warmer oceans of the future.

Who or what will benefit from these outputs, and how?

The outputs will benefit researchers, both within our research group and more widely in the field as well as policy makers, conservation groups and government organisations, both on the national and international scale. The target species themselves will benefit through improved ability to conserve populations.

How will you look to maximise the outputs of this work?

The scientific information produced during the course of the project will be widely shared on an international level including being published in open-access journals, presented at scientific meetings and available to other researchers within the field.

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.

The species of fish chosen are of conservation importance and existing data on their behaviours are lacking, particularly in relation to environmental variables such as oxygen.

Different species of fish will be tested. Comparing different species with different life-histories, physiology and behaviour (e.g. predatory vs filter-feeding; mesotherm vs ectotherm) in addition to threat status will provide a deeper understanding of how fish essential habitats - supporting different fish lifestyles - are altered by changing environment.

We have chosen adult life stages in order to collect data on the full repertoire of behaviour including foraging movements, feeding behaviours, home range including areas supporting reproduction, habitat selection and migratory pathways.

Typically, what will be done to an animal used in your project?

Fish will be fitted with an external telemetry device on their dorsal fin which records their movements as well as environmental variables. Fish larger than 3 metres in length will be tagged while free- swimming while fish between 1-3 metres in length will first be caught by the most humane method available, physically restrained in the water alongside the vessel and fitted with the device before being set free to the wild. The telemetry devices are designed to fall off after a period of days to months.

Typically, animals will experience mild pain at the tagging site during the procedure and for up to several hours after, and no lasting harm.

What are the expected impacts and/or adverse effects for the animals during your project?

All fish undergoing the procedure are expected to experience mild pain during the attachment of the external telemetry device and it is expected that there may be some continuing mild pain at the tag site for several hours. Previous studies have shown that fish return to normal patterns of behaviour within six hours of similar tagging procedures, so the adverse effects are not expected to persist beyond this point.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severity for all animals is expected to be 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 purpose of this programme on fish behavioural ecology cannot be achieved by any other practicable means than using whole, live animals. To understand how wild fish respond to natural and human-made environmental changes it is necessary to study whole, living fish in their natural habitats.

Which non-animal alternatives did you consider for use in this project?

We considered the use of computer models.

Why were they not suitable?

Computer models are not suitable because the preliminary data required to design a suitable model has not yet been collected.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 undertake natural at-sea experiments on how fish move and behave in relation to environmental variations. This requires a sufficient number of animals to be tagged to deliver a minimum number of appropriate datasets. By its nature this will not necessarily be a balanced design but previous published work demonstrates >20 temporally long datasets per species within a region studied (e.g. eastern North Atlantic Ocean) are needed to capture the range of movements and behaviours present and to test hypotheses adequately. Previous published work using computer simulations of marine animal spatial movements to understand effects of sample size on extent of behaviour types observed demonstrate that 75% of migration target locations were identified from tracking at least 40 individuals. The numbers of fish we propose to use reflect this experience.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Return rates of tags from which data will be successfully downloaded are high and close to 90% of those deployed. Knowing this we can limit the number of fish tagged that will be necessary to return datasets necessary to achieve statistical power. In addition, we use satellite data-logging tags that have high data reporting rates via satellite relay so fewer fish need to be tagged to achieve the desired number of appropriate complete datasets. We also chose satellite tags which are designed to fall off after a set period of time to eliminate the requirement to recapture animals.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We conducted pilot studies in the Azores and Cape Verde. We will take the data produced and produce computer models to reduce the need for further animals in the future and

share with other researchers.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The fish species we will study have been chosen because they are of conservation importance therefore the methods we will use have been selected most carefully to minimise any pain, suffering, distress or lasting harm. The procedures are mild and are used in our objectives to study the behaviour of fish that are healthy and not physiologically compromised.

We chose to conduct tagging at sea as this greatly reduces the stress and potential for harm compared to bringing animals into captivity. It is not possible to breed these animals in captivity. Fish larger than 3m in length can be successfully tagged without needing to be captured, reducing the potential for stress or harm.

The tagging methods we will use have been chosen based on our years of previous experience and success in retrieving data from healthy, growing fish after periods of over 1 year. Our methods for attachment of external tags are designed to be rapid and low-impact such that they can be undertaken without anaesthesia which in itself poses a very high risk of distress and lasting harm in elasmobranch fish due to poor induction and recovery times. Compared to tagging alone, injectable anaesthetics would also cause a similar amount of pain to the animal due to the need to use wide gauge needles and thus have been avoided.

Fish 1 - 3 m in length will be restrained alongside a research vessel but not removed from the sea and will have a continuous or intermittent flow of clean seawater passed over their gills because this helps regain normal movements and behaviour faster following restraint. Before tags are attached externally in fish 1 – 3 m in length the body surface for attachment will be treated with a topical antiseptic. This will help reduce the chance of post-tagging infection and should contribute to minimising lasting harm to the animal from external tags. The fish will also be held in a position with the ventral side up, this reduces movements and appears to provide a calming effect, known as tonic-immobility.

Why can’t you use animals that are less sentient?

It is not possible to gain understanding of fish behaviour and ecology, and specifically regarding the responses of these species of conservation importance to natural and human-made environmental changes, by studying any other species which are less sentient.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have refined our capture, restrain and tagging procedures over several decades of successful project work. Post-procedural care and pain management is not possible due to releasing the animals to the wild. Advice and involvement of the NVS and NACWO will be sought as appropriate to identify further refinements during the project. Working with the NACWO and NVS we will produce species- specific health and welfare charts to assess the impacts of the procedures during real time.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

There are best practice guidances produced by NOAA and WWF which we have used to assist with planning and will continue to use during the project: https://www.fisheries.noaa.gov/new-england-mid-atlantic/atlantic-highly-migratory-species/tagging- instructions-and-resources-volunteers

https://sharks.panda.org/images/RAT/pdf/WWF_RAT_Tool5_TaggingTracking.pdf

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 sign up to the NC3Rs newsletter. We will continue to remain in close contact with the NACWO and NTCO regarding any published information about advances in the 3Rs and work together to implement these advances.

48. Neutrophil-T cell interactions in inflammatory 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

T cells, Neutrophils, IL-17, Cancer, Inflammation

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To understand the impact of neutrophils on the development of T cell responses, both in healthy physiology and during inflammatory 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?

Neutrophils and T cells are two of the most abundant cell types in humans, and they interact frequently during health and disease. We know from work done by us and by others that this has profound effects on the phenotype and behaviour of the T cells.

Dysregulated T cell responses are the driving force behind a large number of autoimmune conditions, including Multiple Sclerosis, inflammatory bowel disease, rheumatoid arthritis, and psoriasis. These diseases have enormous impacts on patients, their families and the NHS. For example, Multiple Sclerosis (MS) is a focus of the research in this project. MS is a neurodegenerative disease which involves progressive development of symptoms including pain, fatigue, vision problems, and difficulty walking and moving. 1 in every 400-500 people is at risk of MS, and Scotland has the highest rates in the world, for reasons we don't completely understand. Life expectancy is 5-10 years lower for a patient with MS than the general population.

Inflammatory bowel disease (IBD) is another disease which this project will investigate. Rates of IBD are continuing to rise, and it now affects close to 1 in 100 people in the UK, meaning a huge impact on the NHS. This group of diseases (comprising ulcerative colitis and Crohn's disease) has enormous impacts on patients, with symptoms including pain, incontinence, bleeding and anaemia, and severe weight loss. 70% of patients with Crohn's disease will need surgery at some point.

We know that T cells are one of the important factors causing MS and Crohn's disease, as well as other autoimmune diseases. Current therapies for autoimmunity target T cells and have been fairly successful. However, some problems remain. Not all patients respond well to the treatments, and response tends to wane over time, meaning new therapies are always needed. There are often also significant side effects. Blocking all T cells from the circulation, as some therapies do, means patients are very vulnerable to serious infection. There is therefore an unmet clinical need to understand how T cells develop, what interactions they have with other cells, and how they are driven into pathogenic behaviour. This is key to developing novel therapies for autoimmunity and improving the ones we have now. We believe that understanding how T cells interact with neutrophils is an essential part of this approach. We propose that understanding how T cells interact with neutrophils is an essential part of this approach and it is this aspect of autoimmune disease we focus on.

What outputs do you think you will see at the end of this project?

The project will yield a large amount of novel scientific data. This includes a mapping of T cell / neutrophil interactions in healthy physiology and during a number of inflammatory diseases; in-depth analysis of T cell activity, survival and cytokine production in a number of tissues during disease; and a spatial analysis of interactions between the cell types in lymph nodes in particular. This information will be published.

Protocols will be developed and optimised throughout the project. These may relate to *in vivo* work (for example optimum methods for isolating neutrophils from mouse brain tissue) or may be *in vitro* protocols (for example optimised staining panels for confocal microscopy).

Novel GA mice will be generated throughout the five years of the project.

Who or what will benefit from these outputs, and how?

The data generated will be published and will be of interest to T cell immunologists, neutrophil biologists, clinicians helping patients with autoimmune diseases, and anyone interested in developing novel therapies to control dysregulated T cell activity. It is likely that immunologists will use the data generated first, with considerable work in patient groups to follow before clinicians will be able to use the information generated. In this way

the clinical use may not occur until after the project has completed.
Protocol and method generation, and novel GA mouse strains, will be of immediate benefit to scientists worldwide.

How will you look to maximise the outputs of this work?

All data will be published in peer-reviewed open access journals. Data which does not lead to publication will be released on request, on BioRXiv, or on the lab website. In this way all knowledge gained during this project will be shared with the maximum number of people, whether or not it leads to high-impact publications.

The data will be presented at internal seminars, at other universities, and at national and international conferences. These presentations will be given by all members of the lab team (PI, PhD students, MSc students, and postdoctoral fellows).

All new and optimised protocols will be released on protocols.io or an equivalent free, open-access methods sharing site, as we have done previously. Novel methods will be published in open-access peer-reviewed journals. All protocols and methods will be linked to on the lab website. We welcome anyone to the lab to observe and learn our protocols.

New strains of mice generated will be deposited in the EMMA repository. Live mice will be shared with anyone who requests them.

Species and numbers of animals expected to be used

- Mice: 9750

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 is informed, in the first instance, by human physiology and disease and we perform a large number of experiments on human cells and tissues. However, understanding the mechanisms underpinning our observations is not yet feasible in human samples. In particular, it is not possible to analyse brain tissue from patients with Multiple Sclerosis until post-mortem, and it is not ethical to repeatedly take cerebrospinal fluid for scientific reasons. In this case therefore, using a mouse model of MS is essential in order to understand what immune responses are occurring at the earliest stages of disease. In other projects, we use many tissues donated by patients but this is often treated in a suboptimal way. For example, tumour tissue is fixed before donation so that it can be examined by a pathologist. This means that *ex vivo* untreated tumour tissue is not available from patients and using mice is the best way to examine it.

In addition, being able to generate GA animals with a gene deleted for particular T cell or neutrophil mediator ensures that mechanisms of disease can be deeply understood. The use of conditional depletion of cells, or transgenic T cells which only respond to one antigen, means we can unravel the mechanisms of disease development at a molecular level.

Mice are the best model animal to use for this project. Their similarity to the human immune system is well understood - in particular, T cells from mice and humans are very similar. Therapeutics currently in use for MS were generated using the mouse model of MS, experimental autoimmune encephalomyelitis (EAE), re-enforcing the utility of mice for these studies. Their genetic tractability and the huge range of resources available for study makes them far superior to rats for this project. In particular, there are enormous numbers of GA mice available to study the immune system. Drosophila or zebrafish, while useful for studying the innate immune response, are not suitable for T cell studies or longer-term studies of chronic disease.

Adult mice will be used in experimental protocols (Protocols 2, 3, 4 and 5). We are interested in understanding how chronic diseases such as MS and solid cancers are triggered in adult patients. As such, using neonate or juvenile mice is not suitable.

Typically, what will be done to an animal used in your project?

The typical mouse in this project is a GA mouse bred under Protocol 1, killed by schedule 1 methods, with tissues removed for *in vitro* analysis and culture. This process accounts for more than two thirds of the animals used in our projects. Of the mice that are used in other protocols, we anticipate approximately one third will be used in protocol 2, one third in protocol 3, and the remaining third will be used in protocols 4, 5 and 6.

For Protocol 2, the typical experience will be a mouse injected with transgenic T cells before being inoculated with antigen in adjuvant. This mouse therefore experiences one intravenous and one subcutaneous injection. These experiments typically last for 7 days during which the mouse is monitored daily. A smaller group of mice may have an immunological mediator such as a cytokine or neutrophil peptide depleted during the experiment, which may involve multiple intraperitoneal injections throughout the experiment timecourse.

For Protocol 3, the typical experience will be a mouse injected with myelin in complete Freund's adjuvant, followed by pertussis toxin, which develops signs of EAE illness. This mouse therefore experiences one subcutaneous and one intraperitoneal injection. These experiments can last up to 28 days but the usual time for a mouse to be killed is on days 10-14. A smaller group of mice may have an immunological mediator such as a cytokine or neutrophil peptide depleted during the experiment, which may involve multiple intraperitoneal injections throughout the experiment timecourse.

For Protocol 4, the most likely experience for the majority of mice is to be injected with tumour cells, and for the tumour size to be monitored over the next 14 days. These mice will therefore experience one subcutaneous injection. A smaller group of mice may have an immunological mediator such as a cytokine or neutrophil peptide depleted during the experiment, which may involve multiple intraperitoneal injections throughout the experiment timecourse.

For Protocol 5, the typical mouse will experience being given DSS in drinking water, after which they will develop signs of colitis. They will typically be killed on day 7 of the experiment. A smaller group of mice may have an immunological mediator such as a cytokine or neutrophil peptide depleted during the experiment, which may involve multiple intraperitoneal injections throughout the experiment timecourse.

For protocol 6, the typical mouse will experience being given an intravenous injection of T cells, after which they will develop signs of colitis. They will typically be killed after 4-6

weeks of monitoring.

What are the expected impacts and/or adverse effects for the animals during your project?

The majority of mice used in this project will not suffer any adverse effects, as they will be used in Protocol 1 for breeding before *in vitro* experiments. Mice used in other protocols do not all suffer adverse events, as a large amount will be used as controls, or will be culled early in the process before illness develops.
However, some mice in protocols 2-6 will suffer adverse events. Expected effects are (but are not limited to):

Protocol 2 - inflammation at the site of injection with adjuvant
Protocol 3 - inflammation at the site of injection with adjuvant; development of flaccid tail; paralysis in hind legs; weight loss; hunching; lack of grooming.
Protocol 4 - adverse effects develop from the burden of the tumour as it grows. These can include weight loss; hunching; lack of grooming; reduction in mobility.
Protocols 5 and 6 - inflammation of the intestines results in weight loss; diarrhoea; bleeding from the rectum; hunching; lack of grooming; reduction in mobility.

Expected severity categories and the 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 - all mice used for breeding (as no GA mice are expected to show a phenotype at breeding stage); all control mice - expected 50% of total mice
Mild - all mice in protocols 2-6 which are culled before signs of illness develop; all mice in protocols 2-5 which experience no weight loss or signs of illness - expected 20% of total mice.
Moderate - all mice in protocols 2-6 which develop signs of illness - expected 30% of total 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?

Our work is deeply rooted in understanding the physiology of human disease, and as such all initial work is performed on human samples (peripheral blood T cells and neutrophils, tissue blocks from pathology departments, and fresh tissue from biopsies). However, not all work can be performed using human cells. In particular, examination of inflammation of the central nervous system is very difficult and highly invasive for patients. Our work also involves understanding very early events in the development of pathology, when patients

are unlikely to know they are ill and so will not be attending clinics.

The majority of our work is performing *in vitro* experiments on cells isolated from mice culled by schedule 1 methods. This allows us to understand mechanisms of disease, and generate hypotheses. However, these do not answer questions about the complex interplay of multiple immune cell subsets which form a large part of our work. Therefore, *in vivo* models of inflammatory disease are also necessary.

Which non-animal alternatives did you consider for use in this project?

Cell lines
in silico analysis of previously published datasets
use of human peripheral blood cells

Why were they not suitable?

There are good T cell lines, such as Jurkat cells, which we make use of in the lab. However, neutrophils are terminally differentiated and not genetically tractable, and there is no physiologically- relevant neutrophil cell line.

We are developing expertise in the lab at analysing published datasets of single cell RNA sequencing, bulk RNA sequencing, and proteomic experiments performed on human and mouse T cells and neutrophils. These are proving useful to support and develop our studies. However, they cannot replace experiments in our multiple lines of GA animals, which are relatively rare lines in which few experiments are performed.

A large number of experiments in our lab use cells isolated from human peripheral blood, and these are the foundation of our work. However, it is difficult to model cell migration through tissues or the complex interplay of multiple immune cell subsets *in vitro*. In addition, it is not easy to keep cultures alive and in a physiological state for months in order to examine the impact of neutrophils on memory or exhausted T cells.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We are informed by the numbers of mice used in the previous project licence. However, since that licence significant funding has been won by the research group and so staff numbers have increased, meaning more experiments will be performed.
Numbers of mice used in breeding (protocol 1) are informed by the typical numbers used in current strain breeding. A large number of mice are used in this protocol owing to the multiple transgenic lines in use. In experimental protocols (2-6), the numbers are estimated from group sizes and experimental plans in our funding proposals.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We continually examine our procedures and protocols to optimise methods such that fewer mice need to be used overall. We are also developing totally new protocols in the lab to analyse published data collected by other teams, so that we can bypass experimentation entirely in some areas.

We aim to set experimental group sizes so that data are usable and reproducible, and robustly answer the scientific question being asked, with no wasted mice. We aim to publish all data in accordance with ARRIVE guidelines. We have a robust system of pilot experiments then power calculations in order to set final groups sizes. Control groups are when possible shared between studies and internal controls used (for example, using contralateral lymph nodes as control for tumour draining lymph nodes, rather than other untreated mice).

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Tissues are shared between experiments, such that one wildtype uninfected mouse can serve as a control for DSS colitis (small intestine), EAE (brain) and inoculation (lymph node) by different staff members.This reduces the number of mice used significantly.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Experimental autoimmune encephalomyelitis: this is currently the best animal model we have of Multiple Sclerosis, and multiple drug treatments given to patients were developed using this model. We have refined the usual EAE protocol over the past five years to ensure the suffering is reduced to the minimum possible. For example, we have halved the amount of pertussis toxin used, so that the severity of disease is lowered and onset is slower (such that overall suffering is reduced).

In order to study how immune cells move into and are activated in the central nervous system, it is necessary to allow the model to develop to a point where the animals experience suffering. However, we endeavour to end experiments as early as possible - the majority of our experiments are ended within 14 days. Mice are monitored extremely closely during these experiments and refinements such as softened food placed in the cage are used to lessen their suffering. Water bottles with long spouts so that the mice can easily access water will also be used. We have switched to using commercial kits of pre-prepared myelin in adjuvant. This gives completely reproducible disease where 100% of mice develop the same signs of disease on the same day. As a result, fewer mice have to be used and the variability is lower. This means we can also control the amount of antigen given to the mice and can lower it so that the disease severity is reduced.

DSS colitis: this model is used to understand more about how immune cells drive development of inflammatory bowel disease in patients. We perform careful dose studies in each genotype of mice used as the disease can vary significantly between strains. Pilot studies are used to assess disease course. We very carefully monitor mice to ensure suffering is kept to a minimum.

Development of solid tumours: we use these models to understand how T cells move into tumours in patients, how they are triggered to develop and to become exhausted, and how they produce different cytokines in different situations. Suffering of the mice is limited by our very careful monitoring of tumour development, including frequent measuring of the tumour size as well as weight loss of the mouse. We end the experiments as soon as we can, which is often early in disease course as we are interested in how early T cell responses are generated in lymph nodes.

Why can’t you use animals that are less sentient?

It is difficult to perform T cell studies or analyse lymph nodes in less sentient animals as the cells are frequently not present or are too different to human T cells to be useful as a model. This is true, for example, of zebrafish larvae, Drosophila and C.elegans. The reagents and protocols we need to use to answer our scientific questions are most established in mice.

As we study the development of T cell responses over a time course of days, it is not possible to use terminally anaesthetised mice.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We continuously review our processes and protocols, in discussions within the lab and with outside experts such as animal technicians, veterinary staff and collaborators in other institutions. This enables us to refine our experimental processes in line with best practice.

Refinements have been put in place for the EAE model after extensive discussion. We are now aiming to replace the use of Complete Freund's adjuvant in protocol 2, with an adjuvant which induces less inflammation at the injection site. Pilot studies will be performed, informed by published studies, to determine if we can use other adjuvants in this protocol and still see robust T cell activation.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow the NC3Rs guidance and aim to publish all our work in accordance with the ARRIVE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The establishment runs frequent training days on the 3Rs, and is developing a community of researchers who meet to discuss these issues. In addition, best practice is informed by discussion with collaborators and attendance at conferences in which these topics are discussed.

I am signed up to the NC3Rs newsletter, which discusses the 3Rs in depth.

49. Synaptic organisation of neuronal circuits for perception and behaviour

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Neuroscience, Neuronal circuits, Perception, Behaviour, Sensory-motor transformations

Animal types	Life stages
Mice	embryo, adult, juvenile, pregnant, neonate, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We aim to improve our understanding of the organisation of the neuronal circuits in the brain that are responsible for representing sensory information and give rise to orienting behaviours. In particular, we wish to find out how nerve cells in sensory areas of the brain communicate in order to represent and integrate information from our different senses to guide behaviour, for example, during the detection and localization of behaviourally relevant sensory stimuli. We also wish to find out how these representations are altered when visual or auditory perception improves as a result from learning.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 the neuronal basis for perception and behaviour is in the first instance a matter of considerable fundamental scientific interest. By generating fundamental new knowledge about the structure and function of specific brain areas we will not only advance our knowledge of brain mechanisms in health, but also help understand what may go wrong in neurological disorders which are currently poorly

understood and represent a heavy burden in society, for example schizophrenia and attention-deficit hyperactivity-disorder (ADHD). Indeed, the estimated annual cost to the UK of mental disorders is £100 billion. The difficulty in designing better treatments for neurological disorders has highlighted the urgent need to improve basic knowledge of neuronal circuits. The data collected during this research program will also be used to build, refine and test biologically accurate models of brain circuits. A lasting benefit of this animal research will be to provide computer models that can be used by other scientists in their future research. Such models can generate new hypotheses through predictions and enable neuroscientists to a better understanding of complex neural systems.

What outputs do you think you will see at the end of this project?

The primary goal of this research is to generate new scientific knowledge about the principles of organisation of synaptic connections that underlie information processing in the brain since this is currently poorly understood. Our discoveries will be actively disseminated via academic publications.

Secondary benefits will be of clinical and economical nature.

Who or what will benefit from these outputs, and how?

Primary benefits

This research program is focused on generating new scientific knowledge about the organisation of neuronal (brain cell) circuits that represent sensory inputs to generate complex behaviours, since this is poorly understood. These experiments will provide valuable new insights into function(s) of brain regions which control motivation, movement and higher cognition. With the use of genetically engineered mouse lines to label specific brain cells and the recording of their electrical and chemical activity in brain slices we will map neuronal connectivity and identify general principles that are applicable across brain regions. Using targeted activation and silencing of specific brain cells as well as electrical recordings and imaging approaches, we will directly assess the physiological relevance of single cell and network function in the living brain, where appropriate.

We will publish these data in high-quality peer reviewed journals and disseminate results to a broader audience (other researchers, the pharmaceutical industry and clinicians) through national and international scientific conference presentations, providing benefits to researchers, students and educators. We are also actively engaged in making our data open source after publication where appropriate so it can be used by theoreticians and experimentalists in their future research.
Secondary benefits

Developing a better understanding of physiological mechanisms underlying normal brain function will also provide a basis for understanding how genetic and disease-induced changes in proteins, synapses (brain cell connections) and neurons causes aberrant network behaviour during neurological disorders, which are currently poorly understood and impose a heavy burden in society. According to the Organisation for Economic Co-operation and Development (OECD) “mental health issues cost the UK about £70 billion a year or roughly 4.5% of GDP, in lost productivity at work, benefits payments, and health care expenditure” [1]. More recently, a study calculated an annual cost to the UK of mental disorders of £100 billion [2], while brain disorders cost Europe £800 billion annually [3]. On a global scale, the estimated cost-of-illness in 2010 was US$ 2.5 trillion for mental illness (compared to US$ 0.3 trillion for cancer) rising to US$ 6 trillion by 2030 (The Global

Economic Burden of Noncommunicable Diseases. Geneva: World Economic Forum 2011). The difficulty in designing better treatments for neurological disorders has highlighted the urgent need to improve basic knowledge of neuronal circuits.

Our experimental approaches provide unrivalled breadth of information regarding brain structure and function but are limited to studying animals models such as rodents. This is because the invasive nature of the approaches renders them not suitable for human studies. Understanding the rodent brain however will inevitably lead to better understanding of the human brain. Benefits on human and animal health will occur on the longer term (i.e., > 5 years) as converting knowledge of promising targets for intervention in circuit disorders into drug therapies is a long-term process.

OECD report on costs of mental health in the UK (2012): hhtp://www.oecd.org/unitedkingdom/uk- needs-to-tackle-high-cost-of-mental-ill-helath.htm Fineberg, N.A., Haddad, P.M., Carpenter, L., Gannon, B., Sharpe, R., Young, A.H., Joyce, E., Rowe, J., Wellsted, D., Nutt, D.J., & Sahakian, B.J. The size, burden and cost of disorders of the brain in the UK. Journal of Psychopharmacology. 27(9):761-70. (2013). Diluca, M., & Olesen j. The cost of brain diseases: a burden or a challenge? Neuron. 82(6):1205:8. (2014).

How will you look to maximise the outputs of this work?

To maximise the outputs of this work, I will collaborate with other researchers to gain new insights and perspectives. I will prioritise the effective dissemination of new knowledge by publishing in open access journals, presenting findings at national and international conferences, and using social media to share my research with a wider audience. I will also publish unsuccessful approaches, to help prevent others from repeating unsuccessful experiments, to lead to more efficient use of resources in scientific research and to contribute to the reduction of animals used in research. By prioritising collaboration, effective dissemination of knowledge, and publication of both successful and unsuccessful approaches, I will be able to increase the impact of this work.

Species and numbers of animals expected to be used

- Mice: 8000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use mice for this study because they are particularly suitable for cellular imaging studies and the availability of genetically altered animals expressing genetically encoded fluorescent proteins in particular neurons has enabled long-term imaging of their structure and function (e.g., Holtmaat et al., 2009). We will work primarily with adult mice, the stage at which neuronal circuits are fully developed, that will be trained on specific behavioural tasks and study both neuronal function and neuronal connectivity. In a minority of experiments we study how sensory perturbations during development impact on behavioural responses and on specific neuronal circuits (<5% of animals). In these

experiments animals will be reared in the dark or with low levels of background sound noise for up to 3 months. To ensure that dark reared animals are not exposed to light from birth, pregnant females will be transferred into a blackout cage prior to birth.

Typically, what will be done to an animal used in your project?

Part of the project will involve the raising of genetically altered mice to allow us to express genes to monitor or manipulate neural activity during sensory processing. These animals are expected to be no different in the way they behave from wild-type controls. The behavioural testing procedures we will use to measure sensory abilities 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 temporary weight loss, but this will always be monitored carefully, and extra food or water provided if body weight falls below certain thresholds. The ability of modern techniques to monitor or alter neuronal activity in particular regions of the brain make it possible to carry out almost all this work in a manner that should cause only temporary pain or discomfort to the animals under 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 aseptic conditions, and with appropriate post-operative care. Adverse effects may occur, but the incidence is likely to be low and methods of control (e.g., pain relief) and the most refined experimental techniques will always be used to mitigate them. Chronic implants for recording neural activity or for delivering flashes of light for the purpose of altering that activity are small and lightweight and do not materially affect the animal's quality of life. Animals will be killed humanely at the end of the experiment. The duration of experiments varies between two-months and 15 months depending on the complexity of the behavioural task. Typical protocols include only one surgical procedure over the course of the experiment. In some cases (<20%) when experiments necessitate repeated administration of substances into the brain (e.g., virus or tracer injections at defined time intervals), animals will undergo up to three surgical procedures. Additional surgical intervention may be carried out only if necessary to maintain the implant (e.g., due to loosening of the implant). In a minority of experiments we might need to use transgenic mice with mild neurological disorders. For example, mice with serotonin 5-HT2C receptor gene knock out in specific neuronal types, which have been found to exhibit reduced anxiety related behaviour. Transgenic mice used in this project might only display mild phenotypes but will allow us to assess how alterations to certain genes might impact on sensory processing and behavioural responses.

What are the expected impacts and/or adverse effects for the animals during your project?

Surgical procedures will be carried out aseptically. Peri and post-operative pain relief will be provided; agents will be administered as agreed in advance with the Named Veterinary Surgeon. Adverse effects from surgical procedures such as weight loss are infrequent, and animals make a rapid recovery within two-days. While food or water rationing leads to some degree of weight loss, no adverse effects are anticipated from behavioural tests, but animals will be closely monitored. During habituation to the setup, mice will be taken through a familiarisation phase whereby they become accustomed to the recording apparatus. To minimise stress animals are kept in a dark and noise-free environment. In cases where animals are trained to obtain a reward, food or water intake is monitored during the behavioural test. Animals will receive their daily water/food dose as a reward during behavioural tasks and this amount will be supplemented after the behavioural session. We will follow international best practices as they evolve.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mouse:
Mild - 50%
Moderate - 50%
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?

Because this project investigates the neuronal circuit organisation for multisensory integration and behaviour and how these change during learning, it can only be carried out using in vivo approaches. Moreover, a key aim of this project is to try to account for changes in sensory perception at microscopic level in terms of the underlying circuitry. This requires the use of relatively invasive recording techniques and post-mortem histological measurements, which would not be ethical or practical to carry out in humans.

Which non-animal alternatives did you consider for use in this project?

Following the PREPARE guidelines and RSPCA suggestions, we considered cell cultures, brain organoids, simpler non-vertebrate organisms, mathematical and computer simulations, and studies in human volunteers. We routinely explore this by searching biomedical databases such as pubmed, google scholar, Web of Science. Example databases currently used in our research include https://portal.brain-map.org/explore/circuits/visual-coding-neuropixels and https://neurallatents.github.io/datasets. Indeed, we have secured funding for developing a computational model of the neuronal circuits we are studying that will allow us to replace and refine animal experiments. Additionally, we consider and attend conferences in particular computational conferences such as COSYNE and NeurIPS as well as by engaging with non-vertebrate neuroscience.

Why were they not suitable?

Brain organoids (three-dimensional (3D) cultures of neural cells that mimic the brain) can be useful to study neuronal connectivity. However, the study of how circuits that represent information from our different sensory modalities interact is not feasible since the organoids lack the organs to sense the environment (eyes and ears). Computer modelling does form an important component of our work, but this relies on the information provided by the animal studies. Therefore, while modelling can reduce the number of mice used, it

cannot replace mouse experiments (https://www.eara.eu/post/feature-why-do- we-need-to-use-animals-in-neuroscience-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?

Calculations are 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. Previous and ongoing work from the laboratory have informed animal numbers. We will use approximately 4500 mice over 5 years in procedures other than simple breeding and maintenance. Animals numbers have also been estimated taking into account the need for breeding transgenic animals to study the role of specific neuronal populations by monitoring and/or perturbing their activity. We may breed and/or maintain up to 5000 mice, some of which will be the same ones as in the additional procedures. Due to the statistical nature of genetics, around half of the animals bred under this protocol will not undergo other regulated procedures but will be reintroduced to the breeding stock or terminated humanely.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We are able to keep animal numbers to a minimum by using cutting edge methods that yield large amounts of data and experimental designs that allow multiple measurements to be made from each animal. Current technologies for large-scale neural recording allow recording as much data in a single animal experiment as previously was obtained with dozens of animals, thus substantially reducing the number of animals being used. We will mainly use repeated-measures experimental designs, which will reduce the number of animals needed as compared to standard grouped designs. Furthermore, to complement our statistical expertise gained during more than 15 years of active research, we will consult with biostatisticians.

In general, lines will be maintained in a homozygous state, to limit the number of offspring with genotypes that cannot be used in the experimental settings. If it is unavoidable to use heterozygote breedings to generate homozygotes, littermates genotyped as heterozygous or wild type will be used as age and gender matched controls. In addition, we will use computer simulations to refine our hypothesis.

Indeed, we have recently received funding for a computational project aiming to create a realistic neuronal network model of the superior colliculus, a brain region particularly relevant for the study of multisensory interactions and guiding orienting responses. This model will be used to replace and or reduce animal experimentation when possible.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Computer simulations will be used to guide our experimental design. Simulation techniques to study the structure and function of biological neural networks can predict patterns of connectivity. The predictions can then be used to guide the formulation of hypothesis, reducing the number of experiments.

To achieve our objectives, we will need transgenic mouse lines. In collaboration with the local biological research facilities, we will develop and maintain the most efficient breeding, minimizing the number of animals during breeding. This will include breeding from homozygous breeders to ensure that all offspring have a suitable genotype as well as cryopreservation of embryos to enable breeding only when animals are needed. To further minimize the number of animals, we plan to minimize individual variability by using inbred strains with genetically homogenous backgrounds. To further reduce animal numbers, animals will be efficiently used whenever possible without adverse effects on animal welfare. Specifically, we will minimise the number of animals by maximizing the amount of data gained from one animal whenever possible. All experiments will be conducted in animals of both sexes, unless a scientific reason suggests the use of one sex. Pilot studies will be used to evaluate new substances, experimental tools and approaches. Data from these studies will be used to inform power calculations for larger experiments.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use mice for this project that are sufficiently close to humans to reveal principles of information processing in the brain. Furthermore, rodents have been a long-standing useful model for behavioural studies of learning and memory, which enables us to build upon a large body of research already carried out, and to relate our findings to previous results. Experiments will be performed in both wild- type animals (approximately 20%) and genetically altered animals (80%). A specific transgenic mouse model used in our lab for auditory experiments has a single point mutation on a gene preventing the early onset of hearing loss. Traditionally, in auditory research labs working with C57BL/6 mice, only animals under 3 months of age can be used in experiments, which is the approximate age at which hearing loss onset occurs. By using this transgenic line, we ensure that data collection from a single animal can be maximized, while it also reduces the variability introduced by the variance in the onset of hearing loss.

For behavioural analysis, we have developed a task in which mice pursue a virtual target presented on a screen to collect a reward. The arena's compartmentalised design allows mice to move freely within compartments and access specific compartments only after successfully touching the virtual target.

This design has proven highly effective in reducing the number of sessions required for mice to learn the virtual pursuit task.

In some experiments, mice are held in a fixed position through an implanted head bar. These experiments are necessary to access specific neurons by expression of a fluorescent marker (RFP, tdTomato) while activity evoked by specific visual stimuli will be recorded using GCaMP6, GCaMP7, GCaMP8 signals or signals from other appropriate genetically encoded sensors or to record from neuronal populations through implanted electrodes that are not compatible with freely moving behaviours. Before any procedures in awake, head-restrained mice, they will be habituated to the experimenter and experimental setup. Typically, they will be accustomed to the experimental setups and/or to head fixation for short periods over several days.

Usually, head-fixed animals will be allowed to run freely on a treadmill, a cylinder, a ball or a similar device, which in our experience contributes to reducing stress levels. Rewards might be given during habituation and/or recordings.

We will do all surgical procedures under strictly aseptic conditions to prevent any post-surgery infections. Post-surgery animals' recovery is directly monitored in a recovery chamber until the animal is returned to its home cage. Typically, once animals are returned to their home cage they are observed at least twice daily.

Experiments will be done in awake animals where our previous data and the data of colleagues would indicate that anaesthetized animals might significantly deviate from information processing in the awake, healthy brain. Furthermore, we will typically group-house mice and provide environmental enrichment, which reduces stress levels significantly compared to isolated housing.

With the help of the animal facility staff, in particular the named information officer and the vets, we constantly monitor best practices and new information available in the international literature and on the NC3RS and RSPCA websites (www.nc3rs.org.uk/our-resources, www.rspca.org.uk/adviceandwelfare/laboratory).

Why can’t you use animals that are less sentient?

This project studies the neuronal circuit organisation that supports the representation of sensory information and the generation of motor responses using the mouse as a model system. The complex processes involve higher cognitive functions that may not be present in less complex animals and would not be observed in terminally anaesthetised animals or in neonates that haven’t fully developed their sensory systems. Additionally, mice exhibit a high degree of similarity to humans in terms of genetics, anatomy, and behaviour.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

To refine the procedures, I will first assess the potential sources of harm to the animals, such as surgery, experimental manipulations, or environmental conditions. Based on this assessment, I will implement measures to minimise or eliminate these sources of harm. For example, I will work with the animal care staff and veterinary professionals to provide post-operative care, such as pain management and appropriate medication, to alleviate pain and discomfort resulting from surgical procedures.

To reduce the stress associated with behavioural experiments, all the delivered stimuli are below the pain threshold, and animals will be frequently handled and habituated to the experimental setup over the course of days. Whenever possible, neural recordings will be carried out in freely moving animals using lightweight implants that are easily supported by

the animal. In some cases, head restraint may be needed to enable valid results. In this case, animals will be habituated to the recording setup in incremental steps starting with short durations on the order of several minutes.

The length of behavioural session will depend on the specific aims of the experiments but we will aim to reduce training stages by continuously refining the behavioural training protocols.

Surgical procedures will be conducted under anaesthesia and aseptic conditions to alleviate pain and reduce the risk of postoperative infection. Postoperative pain and inflammation will be closely monitored, typically twice a day. Animals will receive preventive pain killers and signs of stress will be monitored. Prompt action will be taken if those signs are detected (as detailed on individual steps on the protocol of this application).

We will typically group-house animals and provide enrichment including nesting material to increase animal welfare. Refinement measure for animals with implants in order to facilitate access to food or increased available space in the home cage will be implemented whenever necessary.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

I will follow guidance published by NC3Rs, for example webinar and videos:
https://www.nc3rs.org.uk/3rs-resources/refining-rodent-stereotactic-surgeries
https://researchanimaltraining.com/article-categories/aseptic-technique/
I will also follow guidance published in scientific journals such as:
Barkus et al. (2022). Refinements to rodent head fixation and fluid/food control for neuroscience. J Neurosci Methods 381:109705 doi: 10.1016/j.jneumeth.2022.109705.
Finally for specific experimental techniques I will stay up to date with advances in the implementation of protocols in my field, for example:
https://www.internationalbrainlab.com/tools
https://www.protocols.io/view/chronic-recoverable-neuropixels-in-mice-e6nvwjo87lmk/v1

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

To stay informed, I will regularly review the scientific literature and attend relevant conferences and workshops to learn about new developments and best practices in animal research. Additionally, I will consult with colleagues and experts in the field to obtain their insights and guidance. Importantly, I will follow guidance published by NC3Rs, a UK-based scientific organisation that works nationally and internationally with the research community to replace, refine and reduce the use of animals in research and testing.
To implement these advances effectively, I will incorporate the latest 3Rs principles into the design and execution of my research project. For example, I will explore the use of alternative methods to animal testing, such as in vitro models or computer simulations, whenever possible. I will also make efforts to stay updated about development of new analytical methods to reduce the number of animals used in the study and new experimental methods to maximise data collection from each animal.

50. The population ecology of UK seabirds

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

Seabirds, disease, parasite, contaminant, pollution

Animal types	Life stages
European shag (Animal taken from the wild)	juvenile, adult, neonate

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim is to quantify the effects of parasitism and pollutants on mobile seabird populations experiencing climate change.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Wild animal populations experience a wide range of infections from parasites (macroparasites such as stomach worms and microparasites such as bacteria and viruses) that can have deleterious effects ranging from sub-lethal chronic effects that limit reproductive success, through to direct mortality events. These can limit population sizes and increase the susceptibility of vulnerable populations to environmental challenges such as extreme weather events. Because of their high mobility, seabirds have been identified as a species group that warrant further investigation. There is a growing recognition of the

importance of parasitism and disease on wild seabird populations, especially in the light of the recent HPAI epidemic. However, understanding and predicting individual diversity in responses and population persistence is challenging. Furthermore, there are significant knowledge gaps on how wild birds contract and transfer disease. Seabirds are also exposed to contaminants from the leaching of chemical contaminants from waste sites into the coastal marine environment and propagation of these chemicals up food webs. Individual seabirds will vary in their exposure based on their distribution and movements. However, it is unclear what effects these contaminants are having on reproductive success and survival as well as traits that underpin these demographic rates such as foraging performance, body condition and immune function. Furthermore, there is increasing concern about emerging contaminants (pharmaceuticals, personal care products, microplastics) that have not yet been examined in detail. We will explore whether particular individuals are more likely to be exposed to infection and contamination (e.g., males versus females, juveniles versus adults, migrants versus residents) and whether individuals differ in how they are affected. This will allow us to build up a picture of the likely impact of the disease and contaminants on wild seabird populations and is a first step in establishing the most likely patterns of disease transmission and pollutant bioaccumulation in species of concern.

What outputs do you think you will see at the end of this project?

Our research has indicated that parasitism is having significant impacts on seabird fitness by reducing reproductive output, and that pollutants are present in high concentrations. Understanding how parasitism and pollutants are interacting with other anthropogenic drivers such as climate change (including extreme weather events) is vital for predicting population level responses in a rapidly changing ecosystem. Our research looking at interactions between parasitism, pollutants and spatial movements is also important for informing both theoretical and empirical understanding of the interplay between seasonal movements, parasitism and contamination in mobile populations in general. Information is disseminated amongst the scientific community via peer-reviewed publications and conference presentations.

Who or what will benefit from these outputs, and how?

The outputs from this work will benefit researchers in seabird ecology, avian conservation, disease ecology and ecotoxicology. The outputs will aid those tasked with managing the conservation of wild seabirds and regulation of activities that affect these protected species. Furthermore, it will provide valuable insights into those managing wildlife disease and associated risks to humans. Our insights will be of value to the general public and ecotourism operators. All data will be open-access and follow FAIR guiding principles.

How will you look to maximise the outputs of this work?

We will publish our results in peer-review journals and non-academic publications (e.g. natural history society magazines). We will present talks to academic colleagues and non-academic audiences. We will work with key individuals in government and industry in order to maximise input of these findings into policy areas including seabird conservation, disease management and wastewater management.

Species and numbers of animals expected to be used

- : 5500
- : 250

- : 250
- : 250
- : 250
- : 250
- : 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.

Wild seabird populations are exposed to a range of parasites, from macroparasites such as stomach worms to microparasites such as viruses, in common with humans and domestic animals.

Understanding the incidence and effects of such parasites is therefore important for human and animal health. This question has become particularly relevant over the last two years as seabirds have experienced a serious avian flu epidemic. Our understanding of the interplay between exposure and immunity, and what impact that has on effects, is poor. Seabirds are high mobile, and understanding the effects of individuals movements and interchange between populations is a critical aspect of their ecology. Seabirds are also exposed to a range of contaminants from waste disposal sites including legacy (e.g. metals, persistent organic compounds, biocides) and emerging (e.g. pharmaceuticals, personal care products, transformation products and microplastics) chemicals. The occurrence of these pollutants in water and sediment can result in the entry of a mixture of toxicants into aquatic food chains. Exposure to combinations of chemicals released from waste sites could therefore lead to demographic impacts on seabirds at the top of the food chain. There may also be important interactions between parasites and contaminants. For example, an individual that has experienced chronic exposure to contaminants may be more susceptible to disease. Disease and contaminants affect all life stages, so we include all such stages in our project plans.

Typically, what will be done to an animal used in your project?

A bird will be caught and have a ring with a unique combination of colours or letters attached to the leg so it can be identified after its release back into the wild, if one isn’t already fitted. All birds are caught by people fully trained in capture of the focal species. No endangered species are currently within the group of birds to be targeted and all personnel can distinguish species listed in Annex A of Council Regulation 338/97 that lists these species. Once caught, the individual will experience one or more optional steps: a) it will be examined visually and by palpitation of the skin for the presence of ectoparasites, and ticks may be removed; b) a blood sample may be taken from a superficial blood vessel; c) feather samples may be taken (Max 10 breast/back feathers and/or 5 crest feathers and/or 10 cheek/neck feathers and/or a distal third of a secondary feather and/or <5% of total feather volume of max 1 primary feather will be taken); d) a cloacal and/or preen gland and/or oral swab may be taken; e) endoparasite removal (or saline for controls) may be injected subcutaneously/intramuscularly. In some cases, birds may also have a logger attached as part of ongoing monitoring work to track movement, funded by work contributing to environmental monitoring requirements for renewable energy projects. The logger may be attached either to a leg ring or to feathers on the back or tail (depending on

what is most appropriate for the species as specified and licenced by The British Trust for Ornithology 'Special Methods' licences, which the applicant holds). These will either be removed after a few weeks or are naturally moulted off after 1-8 weeks in the case of those attached to feathers. Geolocation loggers weigh <0.5% of body mass and GPS data loggers weigh <3% of body mass and there is no evidence of any negative effects of these loggers on fitness in the populations in which they are used. This technology provides additional opportunity to explore the relationship between movement, infection and contamination around the natural environment. The bird will be handled for a few minutes up to a typical maximum of 20 minutes. It will then be returned to the wild.

What are the expected impacts and/or adverse effects for the animals during your project?

The likely impact on the birds being caught and handled includes elevated stress levels from being caught (up to 20 minutes in the hand, but typically <10 minutes) and temporary discomfort while blood is taken (<1 min), feathers are sampled (<1 min), anti-parasitic drugs (or saline controls) are injected (<1 min) and swabs taken (a few seconds per swab).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild 100%

What will happen to animals at the end of this project?

- Set free

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We wish to measure the behaviour, physiology, pathogen load, contaminant load and demography of individual seabirds, which can only be done in vivo. Although parasite and contaminant load can be obtained from dead birds that are found washed up on the coast, these are not representative of healthy, wild individuals behaving normally and therefore results would be biased and misleading. In addition, our work requires following live individuals as they undertake seasonal migrations and return to the colony to breed. Further, there is no method of capturing the same information in vitro.

Which non-animal alternatives did you consider for use in this project?

Faecal samples; dead birds; environmental sampling

Why were they not suitable?

Faecal samples provide limited information because there is currently no indirect

methodology for detecting certain diseases of interest in (e.g. respiratory viruses where no replication occurs within the gut) or of contaminants that bioaccumulate in tissues (e.g. preen oil, feathers, blood). Some information can potentially be obtained about particular pathogens from dead birds and from environmental sampling. However, sampling dead birds for specific pathogens provide information on those that are susceptible and have died, but not a full understanding of the future impact of infection, which requires information on the proportion of individuals that have been exposed to infection and contaminants. This is also the component of the population that can potentially play the primary role in disease transmission.

Environmental samples provide insights on disease presence in an area but cannot tell us about individual responses to infection that is needed for modelling future disease scenarios and mitigations.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Prevalence levels can change from year to year and differ between populations and species therefore numbers proposed are maximal and are continually adjusted based on minimum likely estimates required based on current prevalence in any population. Further, sample sizes proposed are based on our experience during previous project licences of statistical analyses that were sufficiently robust to enable us to obtain the scientific evidence required with the minimum number of animals used.

Nonetheless, assessing numbers required in a responsive rather than pre-determined mode is one of our continual refinements to minimise the number of birds that require to be sampled. It is also essential due to the unpredictable nature of disease progression in the wild. For example, the current avian influenza outbreak is a key pathogen which has now moved widely into seabird species. We have the expertise and statistical support to assess minimal numbers required to address key questions in this responsive mode.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Sample sizes are based on data collected and analysed under previous project licences that give indicative levels of parasite prevalence or contaminant exposure in different species. Sample sizes will be continually revised if prevalence levels in the wild change substantially. For example, if parasite prevalence is indicated to be higher, then sample sizes can be reduced in order to obtain sufficient data for robust analyses. Individual birds may be recaptured to undertake a similar set of steps up to five times over a three month period, which allows us to study longitudinal change to disease exposure, a key objective. This approach allows us to quantify repeatability within individuals, which we can use to inform future 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?

The power to detect relevant effects is increased if random variation between individuals can be accounted for. We therefore utilise a mixed modelling approach to analyse our data. In particular, typical of pathogen distributions in host populations, and contaminant concentrations in tissues, the deviations from group means are generally not normally distributed. Our statistical framework allows us to specify other error structures, and thus to maximise the information that can be gained for the minimal number of samples. Our study populations have been monitored for over 40 years so there is considerable biological data that can be utilised in the analysis to explain background variation in other traits to increase the power of analyses allowing minimum numbers to be utilised. Where we can, we are sampling birds that are already being caught for other projects, such as tracking work being done to enable marine renewable developments to be sited with minimal impact. This both limits the number of birds being caught and maximises the information gained from a single catch and there is synergy by combining this work as tracking information is also useful in understanding how infection may be moved around the environment.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The rationale for working on these seabird species is that they are model species for addressing these research objectives in the context of understanding drivers of seabird populations. Thus, we have a substantial knowledge of the ecology of these species and a strong background in identifying the drivers of population changes in these species. We also have enormous experience of working with the species concerned in general and with the procedures set out in this application. The methods proposed are the most appropriate for quantifying the parameters in question (diet, pollutant levels, parasite loads). We will maximise the use of each sample to answer multiple questions/objectives. Our experience of analysis data of this kind are reflected in the sample sizes required for each protocol, which has been developed over the course of past project licences. For example swabs taken from the endoscope will be used to measure contaminants. Seabirds are an ideal system for our general approach of establishing the link between environmental change and species' health by studying the behaviour, physiology and demography of individual animals because they show minimal effects of being caught and handled by people, and respond well to procedures such as those set out in this application. Furthermore, we continually seek to minimise animal suffering by reducing handling time to a minimum and adopting handling and procedural methods based on our long experience with the species in question.

Why can't you use animals that are less sentient?

Although some information can be gained from the examination of carcasses, fully

understanding the level of infection and contamination in the population, including exposure and routes of transmission, we need to sample the live bird population. Furthermore, many pathogens and contaminants have sub- lethal effects in wild birds, and understanding the demographic consequences of such complex effects requires the study of live individuals monitored over time and living in the wild.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We are continually monitoring the impact of our field work and associated procedures and exploring ways to reduce handling times. Post handling observation is a standard part of our procedures. We use the data we collect in the field to continually review our proposed sample sizes to ensure sampling the appropriate number of birds for given levels of infection prevalence in the field.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All catching and handling of birds to conduct the procedures is carried out using standard methods as set out by the ringing licensing authority, the British Trust for Ornithology (BTO).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our establishment receives information from NC3Rs.org including their newsletters and events which are useful in keeping up to date with general developments. We also attend scientific meetings and discuss with colleagues in our field, allowing us to keep us up to date about advances that could enable us to refine our fieldwork procedures.

51. Tissue Regeneration and Repair

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Cell transplantation, DNA/RNA delivery, Tissue regeneration, Stem cells

Animal types	Life stages
Mice	adult, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to advance our group's understanding and development of the *in vivo* DNA/RNA delivery in tissues and organs, and test the *in vivo* safety, biocompatibility, durability, and function of bio-scaffolds made from natural, synthetic or hybrid biomaterials for improving tissue regeneration and repair.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Skeletal muscle ageing, neuromuscular disorders, and regenerative medicine challenges

Skeletal muscle normally exhibits robust regenerative potential in youth; however, myogenic activity declines with age, resulting in defective muscle repair after injury.

The combination of age-related skeletal muscle mass loss, defective repair and weakness is a major cause of physical dysfunction in elderly, which is primarily characterized by

mobility impairment, increased risk of falls and fractures, greater hospital, and nursing home admissions, decreased ability to perform activities of daily living, disabilities, loss of independence and social isolation (Tieland et al., J Cach Sarc Muscle 2017). The direct health and social care spending including loss of mobility and frailty for those aged ≥65 (12 m) in the UK was approximately £15bn in 2020 with a predicted increasing trend as life expectancy has increased and the pace of ageing is accelerating, so does the global trend (https://www2.deloitte.com/uk/en/pages/life-sciences-and-healthcare/articles/better-care-for-frail-older- people.html). Age-related skeletal muscle defective repair is associated with multiple comorbidities including bone loss, metabolic rate reduction, fat gain, diabetes, metabolic syndrome, and heart diseases.

Among muscle wasting disorders, neuromuscular diseases as well as illnesses that affect systems closely related to skeletal muscle also remain unresolved as we poorly understand their mechanisms of action (Lovering et al., Phys Ther 2005). Muscular dystrophies (MDs) are one major class of genetic neuromuscular disorders affecting the musculoskeletal system and corresponding molecular components and processes with worldwide reports of incidence of 1 in 200,000 to 1 in 350,000. MDs are a genetically and clinically heterogeneous group of progressive, rare, genetically inherited muscle- wasting diseases, affecting primarily the muscles surrounding the shoulder, hip and thigh bones (i.e. limb girdles) and in some subtypes, cardiac and respiratory muscles. The result is a progressive decline in muscle function because of necrotic muscle fibre loss, enhanced inflammatory infiltration, and development of fibrotic tissue (Moore et al., J. Neuropathol. Exp. Neurol. 2006). The differences among subtypes have prevented the adoption of standardised therapeutic interventions, and there are no approved disease-modifying therapies. Current treatment strategies focus on maintaining skeletal, respiratory and cardiac functions, maximising the quality of life.

Direct transplantation of stem cells, gene therapy, or nanoparticle delivery system represent promising therapeutic options for muscle wasting in neuromuscular disorders and ageing and provide an effective source of tools that can repair organs and tissues in response to future challenges. This studywill allow us to gather further preclinical data in the optimisation of DNA/RNA delivery techniques, and in the evaluation of improved candidate biomaterials, cell types and cell seeding strategies. By applying these regenerative medicine principles, we hope to facilitate the regeneration of viable injured/diseased or aged organs and tissues. These approaches could be translated to humans if shown to be successful in pre-clinical *in vivo* models.

What outputs do you think you will see at the end of this project?

Overall, this project will allow us to build on our group's extensive *in vitro* and *in vivo* work, to focus on testing which combinations of cells, DNA/RNA delivery techniques and biomaterials are likely to be most successful clinically.

In our organoid programme of work, we have been successful in producing an *in vitro* product that reproduces the microscopic structure of a human native musculature and brain.

Thus far, translational applications have to be delivered with 'best guess' specifications for cell types, seeding and timing - this particular project will allow us to optimize these parameters to enable maximal cell survival, tissue regeneration and repair. This data will in turn form an integral part of the pre-clinical justification for full-scale clinical trials, which are essential for cell and gene therapy to become an established treatment modality.

Moreover, the *in vivo* interplay between cells and biomaterials remains a largely unchartered area and is best addressed in a relevant *in vivo* model. International ethical and regulatory approval for more widespread clinical trials is heavily reliant on the further developments of preclinical data demonstrating progression from increased understanding in animal studies to treatment evolutions in clinic and are likely to show improvements in safety and efficacy. Information gained from these experiments will therefore give valuable indicators.

Who or what will benefit from these outputs, and how?

Great leaps have been made in the fields of regenerative medicine and transplantation strategies over the last decade. The prospect of being able to induce regeneration in tissues and organs is a hugely attractive one and would obviate the need for human tissue donors. However, many scientific questions remain to be addressed before the technology can be universally accepted.

There are two main outputs from the studies herein:
Short-term benefits would be a greater understanding of the regenerative capacity of tissue- forming cells/progenitor cells, including those transformed by viral vectors or nanoparticles to express desirable genes to facilitate stem cell expansion and regenerative potential. Understanding of the mechanisms of biomaterial biological integration and tissue repair is of great value. Such studies, including work on angiogenesis, immune response, cell differentiation, migration and persistence of implanted cells and paracrine effects of such cells, not only add considerably to the body of knowledge in this and related fields, but also generate hypotheses for quality improvements in the design and production of second- (and later) generation products.

In the long-term, benefits would be improved treatments for those with neuromuscular disorders and ageing-related diseases.

How will you look to maximise the outputs of this work?

A multichannel communication strategy would allow the capitalisation of existing and new relationships within academic circles, patients' associations, healthcare professionals, industry and funders through a combination of digital information dissemination that needs to be dynamic, relevant, informative, personalised and custom-made for the targeted audience(s); the publication of scientific articles, engagement with diversified media platforms, and in-person engagement by attending scientific and partnering conferences, networking events and giving and attending talks.

Knowledge and hypothesis generation relating to the delivery of cells or genes to the damaged or diseased tissue. Intellectual property generation. Publications in high impact basic science and translational journals in combinations with the in vitro data. Further, GLP, large animal, Project License application based on all the above.

Species and numbers of animals expected to be used.

- Mice: 1550

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures,

including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mouse is suitable species for use in these studies because is the only species for which an extensive collection of strains, including genetic mutants, disease models, and transgenic/knockout animals, is available and vital to the proposed studies. In addition, established methods for the purification of muscle stem and progenitor cells exist only for this species. Murine models with genetic diseases are the appropriate species for the delivery of cells/genes, as they are suitable for modelling of human conditions. Therapeutic treatment will be executed after birth at earlier or later developmental life stages to investigate changes in the disease 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 (combinations of cells, DNA, or RNA) by one injection using standard routes (intramuscular, intraperitoneal, intravenous, or subcutaneous). Endurance and muscle strength measurements: rotarod (overall motor function), grip meter (forelimb strength), inverted cling (strength/endurance), and voluntary wheel running tests are simple non-invasive methods designed to evaluate muscle force in vivo and commonly used for monitoring impaired limb strength (fore and/or hind limb) caused by pathology progression and/or chronic exercise in WT, diseased mice and for determining whether therapeutic interventions (drug, gene and/or cellular) can reduce muscle weakness. All mice will be kept alive for 24h, one week, one month or one year and then killed for tissue analysis.

Subcutaneous or bilateral intramuscular (hind leg) bio-scaffold implantation will be performed under appropriate, inhalational anaesthesia and analgesia. Animals will typically undergo a 5- to 20-minute procedure for the bio-scaffold implant. Limb skin incision and creation of 0.5-2mm pocket(s) inside the skeletal muscle(s). Insertion and securement of the bio-scaffold within the pocket(s). Closure of the muscle pocket(s) with sutures by an appropriate layer-by-layer technique, and the skin with sutures.
These animals will experience some discomfort after surgery and some mild to moderate pain which will be treated with analgesics. After implant, animals are often active, eating and drinking, and tend to remain active throughout the experiment. At the end of the experiment (24h up to one year), animals will be killed by a humane method and tissues taken for analysis after death.

What are the expected impacts and/or adverse effects for the animals during your project?

Potential immune response to the injected substances is rare. Where animals are to receive allogeneic or xenogeneic genetically manipulated or unmanipulated cells or viral vectors alone, either in isolation, or in conjunction with biomaterials and/or bioactive factors, immunodeficient animals will be used.

Genetically immunodeficient mice will be generated or purchased to avoid rejection of cells or vehicles carrying human genes.

Animals will have minor surgery using aseptic technique to implant bio-scaffold under the skin or intramuscularly. They are expected to recover rapidly and will be given painkillers and post-operative care and monitored daily.

Adverse Effects
Anaesthesia*:* it will be monitored continuously by pedal reflex and observation of respiratory rate. Action: Cease operating and deepen anaesthetic. *Humane Endpoint:* immediate termination if signs of continued pain/distress persist on advice from NACWO and/or NVS.
Pain/distress: Mild-moderate pain - common. Daily inspection by competent person using mouse- welfare sheets-appropriate system. *Action:* Administer analgesia as recommended by NVS. Termination if signs of continued moderate pain/distress persist beyond 6 hours. *Humane Endpoint:* immediate termination if signs of continued pain/distress persist on advice from NACWO and/or NVS.
Loss of skin sutures/clips/wound dehiscence: - 1 in 30 - visible on inspection. Action: Wound toilet appropriate to the type of wound and re-suture of uncomplicated, uninfected wounds under general anaesthesia once if animal otherwise healthy/well-recovering (up to once per animal if advised by the NVS). *Humane Endpoint:* termination if dehiscence recurs following re-suturing or if signs of wound infection.
Persistent weight loss approaching or likely to exceed 15% total body weight: Not expected; visible on inspection, daily weighting. *Action:* Termination.
*Infection at surgical site or in the overlying skin wound: N*ot expected (1 in 50); Mild: redness/swelling at wound edges or immediate surrounding skin only. *Action:* Humane Endpoint: immediate termination if implant rejection/loss, signs of infection.
Licking/biting own or other animal's wounds: Common; wound inspection. *Action:* Mice will be individually housed if biting each other. If chewing their own, the NACWO/NVS will be contacted, and the wound may need to be repaired (only once) or the mouse 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)?
Breeding and maintenance of GA mice 100% sub-threshold.

Administration of substances by injection using standard routes: mild, transient pain (90% of the mice). Animals subjected to bio-scaffold implant: moderate (10% 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?

Neither *in vitro* nor *ex vivo* systems can model the immune response or the *in vivo* milieu with sufficient complexity to investigate cell transplantation or *in vivo* protein distribution in the host's tissue and ensure such deliveries can function effectively when within the body. High standards of laboratory animal welfare are important not only for the sake of the animals used but also because animal welfare can impact on scientific outcomes, including the validity of animals as models of human disease as well as the reproducibility of the

studies. I will minimise the pain, suffering, distress or lasting harm that may be experienced by research animals and improve their welfare.

Which non-animal alternatives did you consider for use in this project?

Alternatives of testing the cell-seeded scaffolds *ex vivo* in bioreactors will be performed prior to experiments in animals. This will inform the conditions of *in vivo* experiments as well as limit experimental groups to include only those that have shown sufficient *in vitro* and *ex vivo* biomechanical, angiogenic and cytocompatible promise. Ultimately, no computer or *in vitro* modelling currently exists that can accurately predict the *in vivo* behaviour of cells and grafts (e.g., PubMed search: *in vitro*, *ex vivo* stem cell engraftment; *in vitro*, *ex vivo* immune response and vascularisation; or bio-scaffold degradation).

Why were they not suitable?

We therefore consider animal use to be unavoidable and appropriate in this experimental stage of the projects described. We have extensive experience with bioreactors and *in vitro* systems that aim to simulate *in vivo* conditions, however these systems cannot model the *in vivo* milieu with sufficient complexity to evaluate complex organs or the *in vivo* integration of cells and scaffolds.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 also estimated from past experiments. Calculations typically show that we need group sizes of 6-8 to achieve the quality of results. We have used our annual return of procedures data to estimate the number of animals that we will need to use for breeding. Experimental groups will be chosen based on experimental principles. Animals treated with nanoparticles, mRNA, cells, biomaterials or their combinations will be the experimental group; conversely, animals treated with the vehicle alone or plain biomaterial will be the control group.

Comprehensive analysis, including tissue analysis, DNA and protein analysis, will be conducted on the harvested tissues from the sacrificed animal groups to maximise the data output. For example, a pilot study will be conducted to test a reagent concentration. To reduce bias, animals will be bred at the BSU facilities or ordered in batches from the same suppliers according to a specified weight. Pairing of experimental and control treatments in each animal is an example of how we plan to use multifactorial designs to minimise bias due to individual variability. Animals will be randomised at the start. Prior to treatment, animals will be randomised to treated/non-treated groups and prepared by a separate researcher, such that the second researcher do not know the treated status of a given sample. Where possible following treatment, animals will be reassigned identifiers to

blind researchers during treatment follow-up and post-mortem analysis. Paired treatments were comparatively evaluated for statistical differences (e.g., using Student's paired T-test), whilst experiments with more than one group will be analysed using ANOVA analysis or similar.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The potential number of experimental animals is based on my previous experience. The animal's usage prediction is quite a challenge, and it will depend on the outcomes at different stages of analysis and how successful is the optimization of the assays.

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., physiological monitoring, *in vivo* imaging, analysis of organs). At the end of the experiment, we will harvest as many tissues as possible at post-mortem. If we don't need to analyse the tissues immediately, we will freeze them and make them available to other researchers working on similar questions.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Inbred strains of mice are the appropriate species for the screening of combinations of cells, nanoparticles and biomaterials, as they are economical to keep and are the lowest species evolutionarily to provide useful data on interactions between the implants and in vivo environment. Syngeneic littermates can also be used to further decrease inter-individual variables. I will ensure the animals are provided with housing that allows the expression of species-specific behaviours, use appropriate anaesthesia and analgesia to minimise pain as advised by NVS, and train animals to cooperate with procedures to minimise any distress.

Mice with implanted bio-scaffolds intramuscularly or under the skin are expected to recover rapidly, and they will be given painkillers and post-operative care and monitored daily.

Why can't you use animals that are less sentient?

Non-mammalian animals such as fish or other invertebrates are limited in their use because they are too different from the human system to provide relevant results. Use of embryos is limited to their immature systems.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

For as long as animals are used in research and testing, suffering must be minimised, and welfare improved, as far as possible by close monitoring, best practise, analgesia as advised by NVS.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

I will follow the ARRIVE guidelines developed as part of an NC3Rs initiative to improve standards of reporting and ensure that the data from animal experiments can be fully evaluated and utilised, and the PREPARE guidelines: http://journals.sagepub.com/doi/full/10.1177/0023677217724823, published to assist with planning animal research and testing.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will regularly check information on NC3Rs website, sign up to the NC3Rs newsletter, attend Regional 3Rs symposia, and have regular discussions with the Named Persons and animal technicians to review current approaches.

52. Understanding the neural circuitry of sensation and how it changes following peripheral nerve injury

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Sensation, Pain, Numbness, Neuropathy, Therapy

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Our aim is to understand how the nervous system processes stimuli applied to the body (such as heating or brushing) so that sensations such as touch, itch and pain are generated. To determine how peripheral nerve injury changes this processing of sensory information and to develop new treatments to promote nerve repair, restore normal sensory function and treat pain.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Sensory disturbance (including both pain and loss of sensation such as numbness) are increasingly common. Peripheral neuropathy which is an important cause of sensory disturbance is becoming more common due to the aging population (up to 14% of the population over the age of 60 have peripheral neuropathy) and the increase of conditions that can cause peripheral neuropathy such as diabetes.

Neuropathic pain which is pain that develops following nerve injury affects almost 10% of the general population. It has a major negative impact on quality of life and unfortunately current treatments are inadequate because they are ineffective and have major side effects such as the addictive potential of opioids. There is therefore a pressing need for treatments to promote nerve repair and treat chronic pain.

What outputs do you think you will see at the end of this project?

We will generate fundamental knowledge into how the nervous system generates different sensations such as pleasant touch, itch and pain.

We will generate knowledge as to how the sensory nervous system changes after nerve injury due to physical trauma, treatment with chemotherapy and diabetes and how this results in disturbed sensation such as numbness and pain.

We will discover potential drug targets which can be used by both ourselves and others (such as the pharmaceutical industry) to promote nerve repair and prevent pain.

We will test new treatment approaches such as gene therapy to promote nerve repair and treat neuropathic pain.

We expect more than 20 publications to be generated from this work program in fully open access journals.

We will generate large scale datasets for instance of gene expression in different types of sensory neurons and how it changes after injury which will be deposited in a database which is fully accessible to others and so can make the best use of our findings for everyone.

We hope to generate patent applications relating to the development of new approaches to treat pain and promote nerve repair.

Who or what will benefit from these outputs, and how?

We hope to benefit the following:

Academics in the field of neuroscience. In the short and medium term we will improve the fundamental understanding as to how distinct neural circuits lead to sensation such as touch, itch and pain. Furthermore some of the techniques that we are developing for instance to silence specific types of neurons could be applicable to other fields such as neuroscience where there is excessive neural activity such as epilepsy. We will openly share the technologies that we develop (such as genetic constructs) with other scientists.

The pharmaceutical industry/biotechnology in the development of drugs to treat pain and promote nerve repair. In the near term by understanding the role of different types of sensory neurons in sensation will be potentially predictive of both efficacy and side of effects of current drugs under development. In the medium term we will have identified novel targets for analgesic drug development.

Patients, the NHS and wider society could greatly benefit in the long term. Developing new, non- addictive therapies to promote nerve repair and prevent neuropathic pain (which affects 8% of the general population) would greatly improve the quality of life of patients,

reduce stress on the NHS and improve participation (back pain alone accounts for 40% of sickness absence in the NHS and overall it costs £10 billion for the UK economy). This is a long term goal (10 years) requiring the translation of better fundamental understanding of the factors underlying poor nerve repair and pain to novel therapies.

How will you look to maximise the outputs of this work?

Collaboration: We collaborate widely including at a national level such as the National Mouse Genetics Network and the Advanced Pain Discovery Platform which also includes clinicians and industry representatives. This will mean that our findings can be adopted both by preclinical researchers in related fields but also translation can be facilitated by early involvement of clinicians and the pharmaceutical industry.

Publication: We will publish positive as well as negative findings. We will ensure that reporting is of a high standard and data will be fully open access so that it is searchable by others.

Wider dissemination: Data will be presented at conferences such as the annual Society for Neuroscience meeting and biennial International Association for the Study of Pain (IASP) meeting. We also take part in public engagement activities.

Professional bodies: Outcomes from our research can also inform policy decisions made by professional bodies which are trying to improve the management of patients with peripheral neuropathy and pain.

Databases: We will upload large datasets of primary data into public repositories such as Gene Expression Omnibus. In addition we will develop our own publicly accessible database which helps visualisation of findings to enhance the impact of our results.

Species and numbers of animals expected to be used.

- Mice: 19500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

This project will use mice. Mice were chosen for this work plan because the sensory nervous system in these animals is similar to humans and shows corresponding changes following nerve injury and so provides a good model. We employ a number of genetic manipulations in order to understand the role of specific types of sensory neurons and the genes they express in sensation. This is made possible by the advanced genetic engineering technology which is available for mice. Because we will be breeding mice to manipulate gene expression life stages including embryo, neonatal and adult animals will be used.

Typically, what will be done to an animal used in your project?

Genetically altered mice will be bred which is not expected to cause any harms. With some

strains of mice, it may be necessary to administer substances that activate the gene of interest. These substances may cause transient weight loss.

Under general anaesthesia animals will undergo surgery in which a peripheral nerve will be partially injured in a controlled fashion which can cause focal weakness of one hindlimb as well as numbness and pain. Pain killers will be given at the time of operation.
Alternative models of neuropathy will include administration of chemotherapy drugs which induce neuropathy or using genetic models which develop diabetes and neuropathy or weight gain due to being fed a high fat diet.
Typically animals will be studied for a month after nerve injury during which time we will test the response of animals to sensory stimuli including: mechanical stimuli eg. bendy hairs (von Frey hairs), thermal stimuli (eg. warming or cooling) or chemicals (eg. capsaicin an extract of chili peppers) which will be applied to the paw. We may also manipulate activity of the sensory nervous system with light (switching it on or off) by applying light to the skin to alter activity in nerve terminals or in some cases (for instance to access neurons that do not innervate skin) following implantation of a light source close to the peripheral nerves.

Animals with diabetes will be studied up to 6 months of age. These stimuli cause transient pain and for any persistent stimuli animals will be able to escape from them. We will also observe motor behaviour for instance in exploring a chamber with a smooth or rough floor surface or whether animals when given a choice have preference for a chamber in which a pain killing drug is given.

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. In some cases surgery to implant a thin cannula to deliver a substance around the nerve or spinal cord is needed. If long term treatment is needed a slow release device will be implanted. These animals will experience some discomfort after surgery and some mild to moderate pain which will be treated with analgesics.
Measurements of electrical changes in neurons as well as imaging to determine brain activity for instance using MRI will be used to assess repair and function of the sensory and motor nerves and their connections within the brain. For these procedures animals will undergo terminal anaesthesia. Animals will be humanely killed at the end and tissue taken after death.

What are the expected impacts and/or adverse effects for the animals during your project?

- Traumatic nerve injury will produce focal weakness of the hindlimb around the ankle.
- All the models of neuropathy may produce altered sensation such as numbness and pain. When a surgical injury to the nerve is performed pain killers will be given at the time of operation.
- The model of diabetes is associated with weight gain and increased frequency of urination and the need to drink more.
- Function of the nervous system is tested by applying stimuli that are predicted to evoke a brief sensation of pain (such as a hot surface) as we are mostly determining the point at which the animals first detect the stimulus. By using stimuli from which animals can withdraw suffering is minimised.
- Some of the drugs used may cause temporary weight loss up to 15% (lasting 1 to 2

weeks).
- Injections to deliver drugs (subcutaneous, intraperitoneal and intramuscular) may briefly cause pain.
- Mice will have minor surgery to implant a device under the skin that can release a medicine slowly or a device to deliver light to control activity of the nervous system. This may be combined with surgery to implant a sterile cannula to deliver drugs to around the nerve or spinal cord. They are expected to recover quickly and will be given painkillers and post-operative 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)?

Overall 60% of animals will be subthreshold, 20% mild severity and 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?

Techniques using cultured neurons are not yet sufficiently advanced that they can fully model the nervous system. For example, pain arises due to the complex interaction of millions of different types of neurons. Although we have a long list of brain regions that become activated following administration of a stimulus that causes pain in humans we do not know which regions are actually important in generating the sensation of pain. This is an important question to answer so that we can correctly target treatments for pain. This is due to the complex connectivity of the nervous system and the multiple cell types involved. Behavioural analysis of gait and sensory function requires the use of awake animals.

Which non-animal alternatives did you consider for use in this project?

- We have pioneered the use of human induced pluripotent stem cells (iPSCs) which can be differentiated into sensory neurons. During this project license these will enable the investigation of molecular interactions and electrical properties of these neurons hence ultimately reducing the use of animals.
- Computational neuronal modelling.
- Experimental pain models in human.

Why were they not suitable?

iPSCs are helpful in understanding the molecular means by which sensory neurons detect stimuli however head-to-head comparison shows that they do not yet fully match sensory neurons in either humans or rodents. Specifically, many subtypes of sensory neurons are not generated by human iPSCs even using current state of the art protocols and both the

pattern of gene expression and functional properties do not yet fully match native sensory neurons. Furthermore, iPSCs can't be used to model sensory circuits as we can't yet use them to look at connections between neurons.

We do undertake computational modelling of the nervous system where we use known information to predict neuronal function. This is helpful in refining our understanding of how neurons work but these models are still predictions which are imperfect even when applied to a single neuron let alone complex connections between thousands of neurons.

Wherever possible we do test normal sensory function in humans and this will continue during the course of this project license however some models require gene manipulation, creation of experimental nerve injury, or modulation of specific brain regions which can't be performed 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?

A bioinformatician in our group helped us determine (using data from our own earlier experiments and published data) the minimum numbers of animals to be used whilst ensuring that the results are statistically significant. Typically, our calculations show that we need group sizes of 8 to 10 (depending on the exact variable being measured) to generate scientifically robust data. 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?

We will use careful experimental design to reduce any bias in our experiments and to reduce variation in the data. We will randomise our allocation of animals to treatment groups and the experimenters undertaking measurements will not know which animals have received the active treatment versus the inactive control treatment (ie. experimenters will be blind). The time of day can impact on sensory function and as far as possible sensory testing will be performed at the same time of day.

Assessment of behaviour will be made in a dedicated, well equipped quiet environment with which the animals have been familiarised to reduce variation from environmental factors. We will examine sensation in male and female animals and build this into our experimental design because both in humans and experimental animals there can be differences in the response of the nervous system to injury and in pain sensation dependent on sex.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

A mouse colony management system will be used to ensure efficient colony maintenance

and reduce the number of animals that we use.

Tissue generated form this project license which is stored in a cryobank and will be available to us for future use (as well as other investigators in our own establishment and also external collaborators).

Pilot experiments will be undertaken in which there is little data for the outcome being assessed in order to design a definitive experiment which is statistically robust.

Our experimental design is such that we will comply with the PREPARE and ARRIVE guidelines.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use models which recapitulate the human condition of neuropathy as closely as possible but are associated with the minimum suffering for the animals. Our intention is for the different animal models to represent different underlying mechanisms generated following nerve injury in patients in order to translate findings to clinical benefit. These will include models of traumatic nerve injury (for instance cutting a nerve) but undertaken in a manner so that weakness to the hindlimb is minimised. Post-operative analgesia will be given. The model of chemotherapy uses the minimum dose to measure changes in sensation but without causing significant weakness and minimise other adverse effects such as weight loss. To model diabetic neuropathy we will use genetic models which are not associated with the degree of toxicity of alternative models which use drugs to induce diabetes and cause a rapid and severe increases in blood glucose and can have direct toxic effects on sensory neurons. These models develop neuropathy around the age of 10 weeks and will be used before the age of 25 weeks to minimise exposure to further complications of diabetes such as the risk of infection and insulin treatment will not be needed. To model the state of pre-diabetes we will use feeding with a high fat diet which causes weight gain but blood glucose does not reach the diabetes range.

We will use genetically altered mice and wherever possible in a manner that enables us to alter gene expression in a very specific way in certain types of sensory neurons in adulthood this will avoid adverse effects of changing gene expression in other cell types in the body or the effect of lacking that gene during development.

Any drugs administered will be done so at the minimum dose to be effective whilst minimising side effects. We will make sure that there is sufficient time between drug treatments so that they do not interact and have combined effects. For long term dosing for instance with an analgesic where possible an osmotic minipump will be used as being more refined than repeated injections. For the use of virus to modify gene expression, subcutaneous injections in neonatal animals will be performed when possible as an

alternative to intrathecal injections in adult animals as it is less invasive.

In assessing behavioural outcomes in relation to sensory function we will predominantly assess the response to threshold natural stimuli (for instance reflex withdrawal to a radiant heat source) rather than subjecting animals to persistent intense stimuli. We will use the most refined means of applying light to study sensory function: if optogenetic stimulation is used in a manner such that it may enhance pain signaling a graded stimulation by starting at the lowest intensity and frequency of light stimulation which will be used to determine threshold. Once threshold is determined mice will be able to choose between a chamber in which optogenetic stimulation is given and a neutral chamber so that if the stimulation is unpleasant it can be avoided. We will also use wireless technology as a more refined model rather than needing to deliver light to the mice via fibre-optic cables.

Why can't you use animals that are less sentient?

The sensory and motor neurons in the mouse have comparable features to those seen in primates, including man and both species show a similar response to nerve injury. Other animals which can be manipulated genetically such as fruit-fly, fish or worms have a sensory nervous system which is very different to mammals and some of the genes involved in mammalian sensation either do not exist in these organisms or have completely different roles so they are not appropriate for this project.

The only way we can gain insight into what a mouse actually senses requires assessment of behaviour in awake mice as we know that anaesthesia suppresses sensory perception. We still have an incomplete understanding of how sensation arises in the nervous system.

Although we can measure activity in select parts of the nervous system under terminal anaesthesia (as we will in this project license) this is informative mechanistically as to how parts of the sensory nervous system function but it can not tell us what an animal actually senses.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals will be closely monitored following surgical procedures for changes in weight, poor wound healing, piloerection, withdrawn behaviour or changes in mobility. If these are observed animals will be treated accordingly (for instance if there is loss of weight wet easily accessible food will be provided), and animals that develop severe effects will be humanely killed. Analgesia will be given at the time of surgery and this may be repeated during the post-operative recovery phase for instance if there is withdrawn behaviour suggesting ongoing pain.

Following treatment with drugs animals will be closely monitored for changes in weight, sedation or changes in mobility. If these are observed animals will be treated accordingly (for instance if there is significant loss of weight dosing will be halted and easily accessible food will be provided), and animals that develop severe effects will be humanely killed.

In models of diabetes animals the weight will be monitored and we will also monitor for piloerection, isolated behaviour, and reduced locomotion. If these are observed animals will be treated accordingly, and animals that develop severe effects will be humanely killed.

When assessing the response to sensory stimuli these will be applied in the most efficient manner to give statistically robust results whilst minimising the number of stimuli. We will

assess naturalistic behaviours in the home cage environment and if these prove robust outcome measures in determining analgesic response these will be used as an alternative to reflex withdrawal to sensory stimuli.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the best practise guidance of:

www.nc3rs.org.uk and www.lasa.co.uk and
www.arriveguidelines.org/

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

- We will:
- Obtain information from relevant websites: www.nc3rs.org.uk
- We also continue to follow local 3Rs information disseminated electronically and attend our internal 3Rs meetings.
- We will continue to liaise with our local NC3R’s regional manager and continue to organise regional meetings on the application of 3R's to pain research which we have previously co- organised and found a very useful forum.

53. Use of mechanical stimuli to enhance drug and vaccine delivery 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

Delivery, Drug, vaccine, ultrasound, cancer

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To improve the amount of a drug or vaccine dose which reaches its intended target within the body. This will improve the efficiency and safety of drugs and vaccines. In particular therapy for solid tumours, where drug delivery is particularly poor due to high intratumoural pressure and dense extracellular matrix, as wells as needle-free delivery of vaccines for respiratory diseases and cancer will be addressed.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 drugs and vaccines could be made more effective and safer if more of the delivered dose reached the target sites within the body. We have developed technology that will help push drugs into solid tumours so that they are treated more effectively or push vaccines across the skin so that they are delivered in a more effective and pain-free way. This

technology uses mechanical stimuli such as ultrasound, or the shockwaves generated by the type of machine used to treat kidney stones or magnetic force to achieve this pushing. We have shown this can work for a range of cancer drugs and some example vaccines, but there are still many classes of new drug and vaccine we need to test and improvements to make to our technology. Ultimately if more drug is delivered to where it needs to go we hope treatment will be more effective. Notably all the technologies we are developing (ultrasound, shockwaves, magnetic force) can be applied non-invasively i.e. without the need for surgery or in the case of vaccine delivery without the need for needles, making the treatments more acceptable to patients. There is also the possibility that combining the new technologies with the old methods of delivery (needles) may be even more effective so this will also be explored. In addition to addressing cancer the work may help us address the perennial problem of flu as well as increasing our preparedness and response to emerging human corona viruses. Furthermore, as these mechanical stimuli have been shown to help stimulate anti-tumour immune responses even when used without a drug we will also investigate the use of ultrasound in this context.

What outputs do you think you will see at the end of this project?

We hope that the technology we test in these studies will be shown to be sufficiently safe and effective to warrant its development for testing in clinical trials in humans and ultimately be used to improve patient care in the surgeries and clinics. We currently have a technology for needle-free vaccination and a technology for enhanced treatment of solid tumours. Our aim is to gather sufficient data that we can initiate a clinical trial for each of these technologies by year 5 of the licence. Our findings will be shared at conferences and in publications and this will help contribute to the understanding of the impact of mechanical stimuli on drug delivery and tumour immuno-biology and response to therapy.

Who or what will benefit from these outputs, and how?

In the short term (2-3 years) our experiments will provide improved understanding of the challenges and approaches to the combination of devices and drugs for enhanced drug delivery. By the end of the 5 years the data outputted will form part of the submission for the approval of early phase clinical trials. Ultimately, improvements in drug and vaccine safety, efficiency and patient acceptance will benefit patients, their families and health care systems. The data we publish will be of use to the academics and clinicians working in the field, helping define links between mechanical impacts on skin and solid tumours and the immune responses which result.

How will you look to maximise the outputs of this work?

We will aim to present and publish our scientific findings (both negative and positive) and ultimately translate our technology into clinical practice.

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.

Testing the delivery of vaccines and the delivery of anti-tumour drugs and the therapeutic response that results involves the immune system. It is therefore important that the test species has a developed immune system which is complete and comparable to that of the human. We will use mice aged between 5 and 10 weeks in age to ensure this requirement is met.

Typically, what will be done to an animal used in your project?

Genetically altered mice, which may be either immunocompromised or most commonly wild type mice will be sourced from registered commercial suppliers. All animals will be held in individually ventilated cages which will protect them from any pathogens in the atmosphere.

Some mice will have tumour cells implanted either under the skin (subcutaneous). Tumours will then be monitored every 2-3 days by either callipers or ultrasound. Ultrasound will only be used on a single occasion for the purpose of tumour measurement.

Some mice will receive therapeutic agents or vaccines by standard routes such as subcutaneous, intravenous, or intraperitoneal injections. Some mice, either prior to or during treatment with the drugs or vaccines, will have their skin (for vaccination studies) or tumour (for tumour therapy studies) exposed to a mechanical stimulus such as ultrasound. This may happen up to three times and over the course of three weeks.

Blood samples will be taken to assess how drugs circulate and if the treatment causes release of markers of toxicity as well as test for successful generation of protective antibodies in vaccinated studies. In experiments looking at tumour therapy tumours will be measured using callipers every 2 to 3 days. In vaccination studies mice shown to have protective antibody levels will be used to test ability to resist a dose of flu. All mice in all experiments will be careful welfare monitoring and recording throughout.

At the end of the experiment which is no more than 90 days the mice will be humanely killed.

What are the expected impacts and/or adverse effects for the animals during your project?

In vaccination studies, control mice challenged with a mild flu strain may experience some mild symptoms of flu such as lethargy, ruffled coat, and weight loss of up to 15%. These symptoms are expected to be transient and fully resolve themselves within 48 hours. Mice which are challenged with pathogen following vaccination are not expected to show any symptoms, as challenge will be dependent on whether an effective level of antibodies has been produced.

Subcutaneous tumours will be allowed to grow to no more than 1000mm3 and on rare occasions may become ulcerated. If the ulcer does not heal rapidly, typically within 24 hours, the mice 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)?

88% Mild, 12% Moderate.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

There are no model systems that can reproduce the complexity of the environment within a solid tumour or the blood vessels that supply it. There are no model systems that can reproduce the structure and environment of mammalian skin with the blood vessels that supply it. There are no models systems that can reproduce the mammalian immune system and how it responds to tumour therapy or vaccination.

Which non-animal alternatives did you consider for use in this project?

We have tried to use discarded pig skin from abattoirs and we are applying for ethical approval to use discarded human skin (neither has working vasculature or complete immune system composition). We are also sourcing skin samples from commercial suppliers.

We have pioneered the use of human liver lobes containing tumour deposits. After these lobes have been surgically resected from the patient we connect them to a perfusion machine and study the ways drugs circulate though the liver and if they deposit in the tumours.

Why were they not suitable?

Pig skin proved to be too variable and neither pig or human skin is connected to the blood supply or immune system so it can answer delivery questions but not questions relating to whether delivery improves the protective effect of a vaccine
The liver lobe model can only be kept alive for 24 hours, so long-term impact on anti-tumour activity can not be assessed. The frequency of surgery on suitable patients means only one of these liver lobes is available per month so we can not produce experiments with sufficient statistical power.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Careful planning and design of our experiments and the use of statistical modelling has helped us estimate the number of mice needed. We used our experience of performing similar studies over the past 10 years to extrapolate the number of control and treatment groups needed and the number of animals needed for each type of study.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We used and will continue to use software such as G-power to optimise (power) the number of animals in each group ensuring statistical significance is achieved. We use the NC3R's Experimental Design Assistant to help optimise design and reduce usage.

Animal models and techniques are chosen that allow information to be gathered from one mouse over a time-course and so a different mouse will not be needed at each time-point, or experiments to be internally controlled by using a treatment and a control tumour on the same mouse rather than 2 separate mice.

By designing in good practice such as randomisation (where animals are randomly assigned to treatment or control groups) and blinding (where the person taking the measurements is unaware of which group received which treatment). This ensures valid reproducible results are obtained and animals are not wasted trying to reproduce flawed experiments. Furthermore, control groups will be kept to the minimum size required to provide significance as informed by pilot studies. Where appropriate, studies will be combined to share control group. When the amount of delivery achieved is being measured several areas can be exposed on one mouse rather than using separate 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 hope that the ultrasound passive cavitation methodology we are developing will allow confirmation of successful delivery without the need for killing of mice in order to section tumours to assess delivery. Once developed and validated this will allow the same animal used in testing of delivery to then be left for testing of efficacy, reducing the total needed.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice are the most suitable model in terms of creating tumours for our cancer work and modelling the immune system for our vaccination work. Tumour sizes will be prevented from exceeding a defined limit (1000mm3). Weight loss will not exceed 15% or more. Typically only untreated controls will approach or reach these limits and these will represent a small proportion of the total.

The technology we use is all applied without surgery from outside of the body and requires only brief (typically less than 10 minutes) of anaesthesia. This means the mice recover quickly and do not suffer pain, distress or lasting harm associated with recovery from surgery.

Our project uses mice, both immunocompetent and immunocompromised, as they are the most suitable model that allows the induction and subsequent treatment.

They are also the most suitable for modelling the human immune system for our vaccine work.

We use two methodologies to achieve our aims, Induction of tumours by subcutaneous injection of cells. These tumours are easily visible and are not prone to metastasis meaning they are less harmful to the animals. We then treat these solid tumours by administrating substances to allow mechanical stimulation of the tumour. These stimulations comprise of stimuli such as ultrasound, magnetic field, or shockwaves. We do not allow tumours to exceed 1000mm3 in comparison to some published guideline maximums of 1200mm3.

We will deliver antigens and/or vaccines using mechanical stimuli and then test the level of protection by analysing blood samples and then will challenge mice with either flu or tumour cells. Control animals in the flu challenge may experience mild flu like symptoms with transient weight loss of up to 15%.

We use both non-invasive calliper measurements, imaging and blood sampling to allow us to check the progress of both the tumour growth and immune challenge. None of our procedures require surgical intervention and being no longer than 90 days, are short in duration.

Why can’t you use animals that are less sentient?

There are species such as zebra fish which have been used to characterise the genetic complexity and development of tumours but the routes of drug delivery that are feasible in fish (dissolution in the water, oral dosing) do not mimic the route we are interested in and the complexity of the tumours and the vasculature that feeds them does not provide a good mimic of the mammalian situation. Fish skin does not provide an accurate copy of human skin and the response to vaccination would be very different.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Combining simple procedures during periods of anaesthesia, for example microchipping whilst anaesthetised for tumour cell implantation reduces the exposure to the anaesthetic and the number of times the mice experience unconsciousness. We hope to continue to develop our technology so that prototype lab based ultrasound transducers can be replaced with more clinically relevant ultrasound probes allowing a move away from exposure performed in a waterbath toward those using ultrasound gel. This will reduce the time taken for the procedure, reducing exposure to anaesthetic.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The Design of Animal Experiments is referred to as well as the Handbook of Laboratory Animal Management and Welfare. UKCCCR Guidelines for the Welfare of Animals in

Experimental Neoplasia. Laboratory science animal association (LASA) website will be used for information and guidance (for example by visiting the LASA open access library of Direct Observation of Procedural Skills). We will use arrive.org and the ARRIVE guidelines therein and the PREPARE guidelines as a resource for planning and performing studies.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Careful tracking of NC3Rs output and website and the attendance at termly animal welfare meetings where concerns are addressed and best practice is shared. Use of the on-line training and record keeping to ensure developments in techniques are shared and adopted by personal licence holders working on the project licence.

54. Antibodies and Mice for Immune Research

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Antibodies, Immunity, Complement, Genetically Altered Mice

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged
Rats	adult
Guinea pigs	adult
Rabbits	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aims of this project are twofold: firstly, to breed various strains of GA mice lacking key immune genes essential for our research programs on dementia and heart disease. Secondly, to generate new antibodies and antisera helping the development of novel treatments and diagnostic tools.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Inflammation and inappropriate action by the immune system have been linked to a host of human diseases, including common disorders affecting swathes of the population such as dementia, heart disease and cancer. However, understanding of the complex mechanisms underlying these conditions remains elusive and if we are to devise and develop new medicines we need a better basic understanding of the diseases together with platforms to test any promising new therapies. To this end we currently have several major ongoing programs of research into the role of the immune system in human disease, these projects include investigations into dementia and heart disease.

The work authorised by this licence is critical to the progress of these research programs (covered by other licences held by the group and collaborators) because they require significant numbers of mice from a wide range of different strains which individually been bred to lack one (or more) genes from the immune system. Furthermore, many of these strains have been bred together with mice which mimic human diseases. The use of such animals is vital, not only to help unpick the complex mechanisms whereby the immune system is involved in disease but also to serve as a test bed for potential therapeutics targeted against specific immune agents.

The aspect of this licence related to the development and production of new antibodies against immune molecules will further support the goals outlined above. This is founded on the versatility of antibodies as research tools which have a very wide range of scientific and medical applications. Thus, the ability to make new antibodies will enhance i) studies of the basic understanding of the immune system, ii) the development of better tests for diagnosis of disease, and iii) the development of new therapeutics.

What outputs do you think you will see at the end of this project?

The primary outputs from the project within the aims stated above, will thus be:

1. Genetically altered mice central to the progress of our research on understanding of the role played by the immune system in disease.

2. New antibodies to be used as novel detection tools and potentially therapeutic agents. These will:

a) help us develop Immunoassays, aiding in diagnosis and monitoring of individuals at risk of inflammatory diseases;

b) serve as potential drug candidate tools allowing testing of novel strategies for immune inhibition in inflammatory diseases.

The mice that are bred and the antibodies we generate during our experimental research programs will produce new information to be typically communicated in scientific publications or presentations (but also engagement with non-specialist audiences). Where antibodies have shown to be particularly useful and novel we may seek to either licence them to companies or apply for patents protecting the intellectual property as we develop them further.

Who or what will benefit from these outputs, and how?

Collaborators (Short/Medium Term): the breeding protocol built into this licence will provide the tools (animals and antibodies) which are critical to enable us and our collaborators to carry out our research work into immune involvement in common diseases.

Scientific Field (Short/Medium Term): the data (eg as communicated in publications) and other outputs (eg antibodies as research tools) arising from the work authorised in this licence will help enrich the scientific understanding of complex diseases and provide tools which we will be willing to share with collaborators (old and new). This will further the overarching goals of our entire research program: to better understand the role of the immune system in disease and develop new therapeutic agents to combat these conditions.

Clinicians/Patients (Medium/Long Term): promising antibodies arising will be assessed in detail and developed with the aim of impacting patient well-being by aiding diagnosis, guiding choice of therapy, and potentially developing new strategies for therapy.

Local establishment (Short/Medium/Long Term): the work generated will be published, maintaining the reputation of the group as a leader in the field. Promising antibodies may be licenced non- exclusively to companies, making them widely accessible and generating funding for the establishment to help with research costs.

How will you look to maximise the outputs of this work?

We have always been and remain absolutely committed to sharing resources – in the case of this licence these are the novel antibodies that we will generate and are happy to provide to collaborators old and new worldwide. We have ongoing collaborations with clinical scientists and the pharmaceutical industry which will continue and will support the impact of the current work. The group has a strong track-record of academic publication and conference dissemination this will also continue across the life of the planned project.

We shall publicise the generation and characterisation of new antibodies through publications, reports and conferences. Publicising and sharing of antibodies in this way helps replace the use of mice that might otherwise be used by other groups to make similar antibodies.

Furthermore, sufficiently characterised antibodies that we have tested will be added to a number of different web based resources such as the SAbDab database (https://opig.stats.ox.ac.uk/webapps/sabdab-sabpred/sabdab/about) curated by the Oxford protein Informatics Group (OPIG). We shall also make new (and existing) monoclonal antibodies available to other researchers through the databases of reagents held by the Dementia Research Institute. This will again help widen knowledge and reduce unwarranted replication using animals of our work.

We are firmly committed to open science, this includes the publication of negative results to ensure that a fully representative impression of the outputs of the work will be widely available.

Species and numbers of animals expected to be used

- Mice: 25,000
- Rats: 180
- Guinea pigs: 50
- Rabbits: 50

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The majority of animals used on this licence (mice, 25,000), will be either transferred to other licences for experimental work related to dementia, multiple sclerosis, myasthenia gravis, heart disease or cancer, or used to maintain the individual genetically altered strains. The mice that are transferred will be typically young adult in age (8 - 12 weeks) of either or both sexes and with the strain being dependent on the particular experimental conditions.

For monoclonal antibody production, mice are well established as the species of choice in most situations; however, it is sometimes necessary or scientifically advantageous to use other species to achieve a good immune response or generate a specific type of antibody to the target protein.

For polyclonal antiserum production, because of the volumes required, rabbits are the species of choice in most situations; however, it is sometimes necessary to use other species, such as rats or guinea pigs, to achieve a good immune response to the target protein.

For all antibody and antisera production, young adult animals will be used, since at this stage their immune systems are well developed and responsive (in contrast to older animals, whose immune systems might be compromised).

In many cases where animals are not required for either breeding or transfer to other experimental licences they may be killed under deep anaesthesia to obtain the maximum volume of blood for purification of immune proteins. Age of animals here may range from young adult to those over a year old (eg ex breeders).

Typically, what will be done to an animal used in your project?

The aims of this project licence will be met using the four protocols which are listed below. For each protocol, a typical example of what an animal might experience is given below.

1. **Breeding and maintenance of genetically modified animals**

2. **Antibody Production**

3. **Blood Products**

4. **Rederivation of embryos into genetically modified strains.**

Protocol 1. Breeding and maintenance of genetically modified animals (mild severity)

The numbers of mice covered by this protocol (25,000) represent the majority of animals to be used during the term of this licence. Here, typically, young adult mice will be paired up according to the standard conventional breeding protocol for genetically altered rodents. Each pair will be allowed to breed freely until they have produced no more than 5 litters, at which point they will be replaced.

To determine genetic status, it will sometimes be necessary to obtain tissue biopsies by one of the following methods: ear punch, blood sampling.

Protocol 2. Antibody Production (mild severity)

A typical experience for animals undergoing this procedure might be as follows:

Animals, (wild-type or genetically altered) will be immunised by a series of injections with the target protein of interest. This will cause no more than momentary discomfort. At certain times animals will have small amounts of blood taken under local anaesthesia (typically for mice this might be 0.02ml), this will cause momentary harm. This test bleed is essential for us to determine the levels of antibodies in the blood. Typically after 2-3 boosts animals will be killed by a Schedule 1 method and the spleens, taken for isolation of specific antibody producing cells (B-cells) and immortalisation in the laboratory (a process of 2-3 months). **Typically for each antibody, 3 mice are immunized, this is to try and ensure that out of each experiment we have a high chance of obtaining the antibodies we need. The number allows for variability in the immune responses between animals and this group size has been established as the most appropriate by over 3 decades of work.**

For the production of polyclonal antisera, typically, rabbits (usually 1 for each polyclonal required) might receive 3 booster immunisations before being bled under terminal anaesthesia.

3. Blood Products (mild severity)

Because we often use animal sera to purify immune proteins for our research we will use this procedure to maximise the amount of blood we obtain from each animal. Thus, wild-type or genetically modified animals will be placed under deep, (surgical level) anaesthesia and then as much blood as possible removed by cardiac puncture before confirming death by a schedule 1 method. Animals used here will normally be those not needed for other experimental procedures or those at the end of their breeding life.

Protocol 4. Rederivation of embryos into genetically modified strains (moderate severity)

Typically in this protocol, female mice will have embryos implanted either surgically or non-surgically into the reproductive tract. The female mouse will give birth to the offspring and treat them as her own. The surgical procedure will cause momentary distress but pain relief will be provided and animals recover quickly.

What are the expected impacts and/or adverse effects for the animals during your project?

In this project we will breed and maintain colonies of various GA mouse strains. We expect these procedures to produce no significant consequences for the mice. While some of the animals that we breed may have the potential to develop a harmful phenotype, e.g., neurological signs, after a certain age, in all cases, they will be killed before reaching that age and before the onset of clinical signs, unless they are moved onto another licence and protocol for a specific purpose (continued use).

We shall also generate antibodies and antisera by immunising animals with proteins of relevance to the research programme. Animals used for antibody production are not expected to experience more than mild discomfort (injections and test bleeds).

We will harvest blood from animals (up to 30 rabbits, 20 guinea pigs, 100 rats and 2000 mice) under deep anaesthesia and thus these animals will experience no more than a minor discomfort.

No more than 50 female mice will be used as recipients for embryos in a standard implantation procedure of 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)?

Mice – severity; mild for most of the animals except for the females undergoing rederivation of embryos which is classified as moderate severity
Rabbits – severity; mild for all animals Rats – severity; mild for all animals

Guinea pig – severity; mild for 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?

The ability to study the deletion or blockade of individual immune proteins in the context of an animal model of disease is critical to several of the research programmes reliant on this licence. The complexity of a multitude of interacting biological systems present in the whole animal simply cannot be reproduced at the moment, either in cellular models or virtually.

Therefore, if we are to make headway towards better diagnostic tools and improved therapeutic drugs in complex disorders such as dementia and heart disease, there is no alternative to the use of these models.

As part of this project, mice and rabbits are key in our endeavours to make antibodies against novel targets of specific proteins that have not been explored before. While there are now some technologies available to make antibodies without the use of animals, the antibodies that are made in this way are usually of low affinity and variable specificity and hence not as effective as those made using animals. Because of this we therefore conclude that it is both necessary and important to be able to use animals for the production of specific antibodies and antisera to obtain high quality tools targeting the proteins of interest. Indeed, many of the antibodies that our group has produced over the decades have been shared widely with collaborators across the globe or commercialised. The fact that we often receive requests for these reagents demonstrates clearly both the quality and the ongoing need for our antibodies, all of which have been derived from the use of animals. Our commitment in making these key research tools freely available to the

broader scientific community hugely reduces the need for other laboratories to use animals for this purpose.

Which non-animal alternatives did you consider for use in this project?

The process of producing monoclonal antibodies has in the past required a significant number of mice in addition to the individual immunised animals. For example, for each new animal immunised for the purpose of making a monoclonal antibody we previously used 3-4 mice during the in vitro cloning phase of the work to provide macrophages (white blood cells) as "feeder cells" which support the growth of the antibody-producing "hybridoma" cells. Recently, we have introduced a refinement that replaces this practice by using new effective synthetic commercially available cell culture supplements. We are currently evaluating one such supplement (BM Condimed H1; Hybridoma Cloning Supplement) and anticipate eliminating the need for mouse-derived "feeder cells". The replacement media are expensive; however, the cost is justified in that we estimate that over the course of 50 separate experiments making monoclonal antibodies we will replace the use of approximately 400 mice.

Current antibody technologies for generating monoclonal antibodies without the use of animals are expensive, require trained staff dedicated to their development and use and present several well-documented disadvantages. An over-arching concern is that, without the complete immune system which oversees, directs and reinforces the generation of antibodies in live animals and humans, antibodies produced in vitro commonly have relatively low binding affinity for the target protein. This is a significant problem for a range of antibody applications, notably in testing potential therapeutic or diagnostic antibodies where high affinity is a necessity. Indeed, the issue of phage display libraries producing antibodies with low binding affinity is an important scientific issue for the wider field concerned with making therapeutic antibodies. Often, testing of such potential therapeutic antibodies requires in vivo use of these reagents to functionally block active proteins of the immune system. If these antibodies do not bind sufficiently tightly to their target then downstream testing (in animal models of disease) will be compromised resulting in wastage of animals and potentially the abandonment of specific therapeutic approaches which might have been fruitful had better quality antibodies with higher binding strengths been used.

Nevertheless, despite these shortcomings, we recognise that there are advantages in the use of in vitro systems, including the ability to "design" or alter many of the features of an antibody to adapt it for specific purposes. Also, we acknowledge that this technology is improving, and therefore during the course of this project licence we shall seek grant funding to develop appropriate systems and produce, test and scientifically compare antibodies generated using fully in vitro library-based technologies with those raised using our well established methodologies requiring low numbers of mice.

Why were they not suitable?

While we have taken antibodies produced through our established methods and modified them using in vitro methodologies (eg to enable better brain penetrance for therapeutic testing), at this current time we have not attempted to either compare or produce novel antibodies by these technologies from scratch. These choices were driven by the documented technological issues and problems detailed above and the lack of grant funding to allow these methodologies to be established and used in our laboratory.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimated number of animals in each protocol is based upon historical usage over the last five years and allows for an anticipated increased activity in antibody production in the group supported by the recent capture of substantial funding. The numbers for breeding of GA animals, reflects the large number of different GA lines that will be maintained and used in the different projects carried out under the authority of this and other licences. The numbers requested represent the minimum numbers required to ensure colony viability and to provide sufficient animals for the various experimental programs described in the Aims section. For each experiment the required number of animals needed is calculated using statistical software such as G*power 3.1.9.4 and the NC3Rs EDA experimental design tool. The numbers are checked with local statisticians.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

While power analyses and the NC3R's EDA tools are certainly extremely useful in the design of experiments testing novel therapeutic agents, those sorts of projects lie largely outside the remit of this particular licence. Here, the protocols for antibody production (the main experimental part of this licence) have been in use for decades, and thus, for monoclonal antibody production, group size for immunisation is selected based upon known properties that influence the effectiveness of the animal's immune response to the protein in question, such factors might include: size, solubility, antigenic differences between species, etc. Proteins predicted to provoke strong immune responses in the immunised species will require fewer animals being immunised by comparison with proteins or fragments of proteins which are expected to give low immune responses. Typically, experience has taught us that a group size of three is sufficient for each separate protein that we are attempting to make antibodies against.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

1. Immunisation protocols **(including group sizes)** have been optimised over many years to increase the likelihood of obtaining a robust immune response and thus reduce the number of animals that need to be immunised for each new protein of interest.

2. **For monoclonal antibody production we shall, wherever possible (timing, availability of antigen etc permitting), seek to immunize animals with multiple target antigens with the aim of screening for a range of different antibodies from the resultant in vitro hybridoma fusion, screening and cloning procedures.**

3. In the downstream in vitro work that is an essential part of producing monoclonal antibodies screening protocols have also been optimised over time to ensure that we have the best chance of selecting the antibodies that we need from each animal immunised, this leads to reducing the number of animals used overall in antibody production.

4. Bulk production of monoclonal antibodies, once requiring ascites production in vivo, is now achieved efficiently using cell culture-based systems.

5. Good bleeding techniques to maximise the yield of blood and blood products and again reduce the number of animals needed to achieve experimental goals.

6. Good colony management, to reduce the numbers required for colony maintenance. The numerous colonies of GA animals required will be maintained at the minimum colony size compatible with viability and supply to 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.

Mice, rats, rabbits, and guinea pigs will be used in this project.

Breeding and maintenance of genetically modified animals

The vast majority of animals used under this licence will be genetically modified mice bred to provide experimental animals for this and other work carried out under other licences. These mice will carry various genetic alterations; generally, these will be deletions of genes encoding complement proteins. However, we may also be breeding strains carrying alterations in genes related to or essential for the use of different disease models, e.g., amyloid mutations (Alzheimer's disease), low-density lipoprotein receptor (LDLR) (cardiovascular disease). Some strains will carry alterations in two (or more) genes. We expect that the strains used under protocols in this licence should experience little or no distress or harm caused by the genetic alterations they carry.

Antibody Production

Mice represent the best species for monoclonal antibody production because of their size, ease of handling, excellent immune responses, and the availability of knockout strains lacking specific proteins of interest. Rats are used rarely and only where a particular protein or protein fragment does not give a good immune response when immunised in mice. Rabbits may be tested for monoclonal antibody production during the course of this project, as the methodology has improved considerably for this species. Polyclonal antisera can be generated in any of the specified species (mouse, rat, rabbit, or guinea pig); the choice is based on predicted strength of the immune response and yield of antiserum. Rabbits are preferred for large-scale antisera production.

Throughout the term of the licence, suffering under each objective and protocol will be minimised by expert handling from our cadre of highly experienced research and technical staff, who will deploy excellent immunisation and bleeding techniques, ensuring minimal harm and no infections at sites of needle puncture. We will have available and seek, wherever appropriate or needed, technical support from experienced animal facility staff. Where appropriate and as stated in protocols, animals will be anaesthetized for some

procedures; this includes the use of local or light general anaesthesia during tail tipping for blood samples to test antibody levels in the blood.

Freund's complete adjuvant (FCA) is used in the immunisations to improve the immune response of the individual animal, however, this substance can cause significant local reactions, although in our long experience, this is very rare with good technique. To minimise the risk of these local reactions, the protein/adjuvant mixture is given in areas of loose skin, and using a combination of volumes, routes and frequencies that of themselves will result in no more than transient discomfort and no lasting harm. FCA is not used more than once on the same animal.

Blood Products

Here, the protocol has been designed to minimise suffering and distress while obtaining as much as possible from each individual animal. Hence, we will ensure an adequate depth of anaesthesia, only proceeding when an animal does not respond to multiple checks of consciousness (eye touch, toe pinch, etc). The animals will be exsanguinated (bled out by direct heart puncture) with confirmation of death by a schedule 1 method. Wherever possible, other tissues may be shared with other research groups (notifications of requirements and availability on electronic animal management software).

Rederivation of embryos into genetically modified strains.

Adult female mice will be used; embryos will be implanted either non-surgically, with or without brief anaesthesia, or by a surgical procedure under general anaesthesia. If embryos are required, the females will be humanely killed by the Schedule 1 method; otherwise, they will be allowed to give birth naturally and raise the litter. The animals will be closely monitored until full recovery following the surgery. If females fail to recover from the procedure or show any signs of failing to do so, they will be killed by a Schedule 1 method.

Why can't you use animals that are less sentient?

Use of adult animals is required as they have a fully formed and active immune system, which is essential for an appropriate response to an immunogen. To be relevant to our area of research into human disease, the antibodies that we will generate during this licence need to be raised against mammalian antigens in mammalian systems. The latter is particularly important to realise the potential of any antibodies produced that may be of therapeutic use.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

For this particular licence the protocols and procedures that will be used are very well established having been optimised, improved and tested by us and many other laboratories (globally) over decades. This does not mean that I and my group are in any way complacent, we will all be alert to opportunities (in the literature and in discussion with technical staff) to improve the welfare of the animals that we are using. One critical area in this regard is the training of new group members, here I will ensure that all such new starters regardless of prior experience are given adequate support until they are proficient in the techniques they are using.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

I will regularly monitor scientific literature for improvements in refinement of these protocols and implement any changes promptly. I will also regularly review the NC3R website for improvements in best practices particularly in case of colony management and breeding of GA animals.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I review the literature regularly for novel methods of antibody generation that can minimise, reduce or eliminate the need to use animals. If and when new advances are developed, I will test and implement them in my home establishment. I sit on one of the establishment's research committees concerned with use of animals. As such I have regular contact with the regional NC3R rep and am able to pass on the knowledge they impart and implement relevant improvements within our own group. This committee also gives me an opportunity to contribute to the establishment’s research agenda, impact local policy, expand my professional network, and explore ideas and initiatives relevant to improving the quality and practices of animal welfare. I am always eager to learn and apply new improvements according to the 3R principles and in addition to the above I regularly survey the NC3Rs website (https://www.nc3rs.org.uk/) for new developments and additional links to other organisations providing information and advancement in the 3Rs.

55. Brain plasticity in development and disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Stem Cells, Brain, Traumatic Brain Injury, Alzheimer's disease, Plasticity

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged
Rats	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to better understand developmental brain plasticity, which is the ability of the nervous system to reorganise its structure, functions, or connections in response to its own stimuli (intrinsic) or stimuli from other parts of the body or environment (extrinsic). We then want to understand how this plasticity is utilised to repair pathology in disease (e.g., Alzheimer’s disease) and injury (e.g. traumatic brain injury).

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Brain injury and disease lead to severe neurological and psychiatric disorders. These disorders can severely diminish the ability of the brain to carry out its normal functions including memory, speech, understanding, emotions, and social skills. Many brain

diseases are associated with aging and the aging population is increasing, thus the societal burden and costs to address diseases such as Alzheimer's disease is increasing enormously. Despite an increase in the ageing population and the central importance of the brain, very few effective drugs or treatments exist that can treat symptoms or eradicate disease. Therefore, the field is actively searching for novel approaches to address this problem. A major effort has emerged called regenerative medicine which seeks to utilise the self- healing properties of the body for recovery from injury and disease.

There are stem cells in most of our organs that participate in normal tissue growth in development, in tissue maintenance and in tissue repair after injury. In the brain this is an important aspect of brain plasticity which includes the ability to respond to pathological environmental insults by growing new nervous system tissue. Brain stem cells are concentrated in two major regions of the brain, the subventricular zone and the subgranular zone. These stem cells become activated in injury and increase the production of nerve cells. However, the repair is incomplete and therefore a major goal is to increase reparation via plasticity. This will be accomplished in this project by understanding the molecular and cellular mechanisms that regulate brain development and repair. Another goal is to guide the stem cells to move to the diseased portion of the brain. Therefore, in another branch of work we will implant exogenous stem cells directly into lesions, to boost their potential for repair. Finally, inflammation regulates both stem cells and repair of damaged tissue. Therefore, it is essential to monitor and modulate inflammation to augment brain plasticity and brain repair.

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 in the following three inter-related topics.

We seek to understand how brain repair can be augmented by activating endogenous stem cells and increasing the production of nerve cells and support cells in the brain. As part of this we are searching for molecules, either natural proteins or man-made drugs that can activate stem cells and increase neurogenesis. Then we seek to reveal to what extent these cells migrate to the injury and help limit or repair the injury. We also assess functional benefits, for example is the ability to remember recovered in models of Alzheimer's disease after stimulating endogenous stem cells.

In a parallel endeavour we seek to understand how stem cells grown in the lab and implanted into the brain can increase repair. We implant human stem cells and their progeny in order to assess how well they anatomically and functionally integrate into host tissue.

Our work will also reveal how reducing proteins, such as Galectin-3 (Gal-3) that normally increase inflammation can augment brain repair and plasticity.

Who or what will benefit from these outputs, and how?

A broad range of scientists, physicians and the public will benefit from this work.

In the short-term a major set of beneficiaries who will soon learn from our work will be developmental neurobiologists. Much of the project is based on stem cell biology, thus stem cell experts will also gain knowledge. Our work will inform and enrich the field of postnatal and adult neurogenesis. Other researchers in our establishment will be informed

of our results via attendance at lectures and seminars. This will result in knowledge being further disseminated as they move to other establishments.

Our findings will also be interesting to the public who have a natural and deep curiosity about the brain. The interest in neurodegenerative disease and mental health has increased dramatically.

In the medium-term, neurologists and psychiatrists will benefit since our data will contribute to understanding of the molecular basis of brain development and brain disease.

In the long-term we hope that patients will benefit from our outputs. This will be achieved in collaboration with other establishments and companies who are looking to develop drugs that increase efficacy of the process of neuronal growth and development.

How will you look to maximise the outputs of this work?

Most of our publications in the past 15 years have involved local and international collaborations. These experts have allowed us to make discoveries that we alone would not have been able to achieve.
Our work will be disseminated via a variety of means, including publications in scientific journals, talks at scientific meetings, seminars for the lay audience. We will also deposit our preliminary publications on publicly available platforms, as we have done in the past. We will also make our raw data available to scientists upon request.

We are firm believer in publishing "negative" results which we believe can be as informative as "positive" results.

Species and numbers of animals expected to be used

- Mice: 8,000
- Rats: 500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We use the mouse for several reasons. Firstly, most knowledge of the molecular regulation of stem cells and mammalian neurogenesis has been discovered in this model organism. Many molecular tools surrounding the mouse models as well as transgenic mice, are available from a variety of organisations and international projects. Mice and humans share many genetic sequences making them a translationally relevant model organism.

We have also included rats in the PPL as they are considered to be more intelligent and easier to train than mice and therefore are an excellent model organism to test cognition and memory. Additionally, the rat has become one of the favoured models for cell implantation as the implants integrate well into host rat brain.

We will use pregnant animals that will be given substances when we wish to manipulate the embryo in utero during different stages of gestation. We will use neonates and juveniles for similar reasons as embryos, because perturbations at these two later time points can have long-lasting effects on brain disease. The majority of brain disease is in adults and thus this period is essential for our work. Finally, we have chosen to work at late life-stages because we are uncovering embryonic developmental principles that are recapitulated in neurodegeneration in aging animals.

Typically, what will be done to an animal used in your project?

Genetically altered mice and rats will be bred. The mice and rats are largely for similar usage, however we will not use embryonic, pregnant or aged rats. Their mutations usually do not have any harmful phenotypes but in some older mice (e.g. that are models of Alzheimer's disease) may do so. In some genetically altered mice other genes of interest can be inactivated by the administration of a substance, typically tamoxifen at various life stages from embryo through to adulthood. Alternatively, they may receive a viral vector on a single occasion to alter their gene expression.

Some animals will undergo surgery to implant a small clear viewing window onto the skull. They may also be fitted with a head post to allow them to be restrained for imaging of the brain once they have recovered from the surgery. The animals will be given pain relief following surgery as you would expect in humans.

We may also implant cells into the brain to manipulate its plasticity and repair. Alternatively, we may inject them at a later stage if we need to first assess the normal brain.

Some animals will undergo surgery to allow a small injury to the brain so that we can monitor the role of genes in repair. The injury can be caused by either a physical method or by chemicals. As above, pain relief is provided post-surgery.

In the large majority of cases we will carry out one cranial surgery, however a few animals may undergo more. We will limit the number of neurosurgical interventions to a maximum of 2 per animal, not including the final terminal procedure.

Whereas most behavioural testing will be in freely moving animals, some animals will undergo behavioural testing whilst being restrained by a previously implanted head post. These later tasks will involve the animals watching a computer screen and responding to objects that they can touch with their large facial whiskers or licking a waterspout in response to a novel stimulus. For these experiments the animals will receive water as a reward, however this means they will have restricted access to water during their non testing hours. Animals tolerate this very well and although they initially may experience a weight loss of up to 15% this very quickly stabilises at around 10% weight reduction from their starting weight. Some animals will also have their brain imaged by microscopy whilst performing these tests.

Due to the need to implant devices into the skull of the animal and at times restrict their water intake animals may be singly housed for a period of up to 4 weeks.

The majority of animals will undergo behavioural tasks to monitor plasticity after brain disease has been established (e.g. memory tests in 5xFAD mice already experiencing cognitive impairment). A minority of animals will experience maternal separation early weaning (MSEW), a behavioural protocol animals experience before brain disease - which induces a model of post-partum depression and post- partum pychosis. This unique

scenario is found only in a small subset of experiments. We will use a variety of behavioural tests including water-escape, the rotarod test and social recognition and dominance testing.

Mice will be allowed to age up to 24 months of age in some cases to allow us to study the effects of ageing on neurological disease such as Alzheimer's. No animal will be tested beyond 24 months of age.

Behavioural testing is designed to test if the mechanisms we have identified as altering SVZ or SGZ stem cell proliferation, differentiation and migration result in behavioural changes. We will test for changes across a broad spectrum of motor skills, cognitive skills, social interactions and emotional status. These data are necessary to gauge how much these molecular mechanisms impact animal behaviour within the models of neurological disease used to achieve Objective 1. They will be complementary to histological examination of the tissue.

The animals are motivated to earn rewards in the experimental apparatus by having their home cage access to food or water controlled. They are thus thirsty at the time they are tested but are not parched and are generally healthier than animals with unrestricted access. Mice will receive a fixed amount of food (e.g. 1-3 g) in order to maintain them at a stable and healthy, lean body weight. Thus, the schedules of water restriction used to motivate appetitive tasks are not predicted to be harmful. Their primary role lies in spacing, so that animals are tested when they are thirsty. Body weight will not be allowed to drop below 85% from the start weight and additional water will be provided if needed. This should be from the start weight (or peak weight) from the initial procedure the animal experiences in order to take cumulative suffering into account. If water restriction is used in growing animals, target weights will be matched appropriately. Where compatible with scientific objectives, additional enrichment such as forage mix will be provided.

We will limit the total number of general anaesthetic doses to a maximum of five per animal to reduce adverse effects. Best practise (administration of appropriate fluid, maintaining core body temp) will be implemented. A small subset of animals might undergo the maximum amount of 5 periods of general anaesthesia. At least 48 hours will elapse between any steps involving general anaesthesia and recovery.

At the end of the experiments, animals will be humanely killed often via perfusion with fixatives and their brains harvested postmortem to allow further investigations in vitro.

What are the expected impacts and/or adverse effects for the animals during your project?

Transient postoperative pain after a surgery is expected and therefore controlled with pain relieving medications. If animals are not feeding appropriately post-surgery dietary assistance with mash will be provided.

Water restriction for our behavioural paradigms can cause mice to lose 10 to 15% of their body weight. This weight loss normally lasts throughout behavioural training and testing which ranges from one week to three months depending on the experiment.

Maternal separation and early weaning (MSEW) one of the behavioural protocols is designed to mimic postpartum depression (PPD) but does not cause adverse effects for the animals that are more than mild and transient. The behavioural tests of water-escape, the rotarod test and social recognition and dominance testing may induce stress in a small number of animals <5%.

Head restraint and fixation are generally very well tolerated by the majority of mice but may induce stress in a few mice. Head fixations may come loose in a small number of mice.

Genetic alterations may increase inflammation in a small percentage of aging 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)?

Mice: 40% Subthreshold, 40% Mild, 20% Moderate
Rats: 40% Subthreshold, 40% Mild, 20% 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 events we are studying - brain plasticity mediated by endogenous or exogenous stem cells and inflammation are dynamic processes that only occur in intact brains. The combinations of cell types, molecular and genetic events in the brain are so complex that they cannot be monitored in vitro alone. It is important to point as well that the goal is to use brain plasticity to recover behaviours such as memory and movement, events involving the whole animal that cannot be modelled in cell cultures.
Additionally, animal models of disease are far more similar to human disease than cell cultures. Finally, it goes without saying that our imaging, behavioural and functional studies cannot ethically be carried out in humans.

Which non-animal alternatives did you consider for use in this project?

We have considered cell culture, computer simulations and post-mortem human histology.

Why were they not suitable?

In vitro studies such as isolated organs, organoids or cultures cannot be used to address the behaviour of animals in these disease contexts as cells simply do not behave in the way that whole organisms do.

Postmortem histology can be very revelatory, but it only provides a brief window of the final stages of life.

At present too little is known about the relevant, physiology, anatomy, and genetics of brain stem cells, plasticity, and inflammation to study these phenomena by computational modelling.

Whereas in vitro, postmortem, and computational approaches have the limitations listed above they can provide a useful complement to inform and validate the animal work. We use all three "non-animal" approaches to study brain development, brain disease, brain inflammation and brain plasticity.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals is based on sample size calculations we performed for typical experiments in preparing the applications funding this work. These numbers are highly consistent with our published past studies. They are also consistent with values from other laboratories performing similar experiments around the world based on literature searches and conferences involving other laboratories. These numbers represent the estimated numbers of experimental animals necessary to generate statistically significant results factoring in the expected fractions of mouse pups that will have the desire transgenes. Our evidence shows that typically we need groups of 6 mice per group to achieve statistical power in our histological analyses and 12 mice per group to achieve statistical significance in behavioural work. We have used our previous home office returns to assess the number of animals required for our breeding programmes.
In the past five years an increasing portion of our lab’s activity has used human post-mortem tissue and human embryonic and iPSCs (induced pluripotential stem cells) thereby decreasing the use of animals.

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 NC3Rs Experimental Design Assistant as a tool for planning our experiments. Large scale recording techniques such as two-photon microscopy can provide data from 10s to hundreds of neurons from a single mouse. This high throughput approach 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 can directly compare molecular data and cellular data with behavioural data.

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 practise 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 and rats 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 majority of genetic mutations we have chosen are not expected to cause any harmful phenotypes beyond mild in severity. 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 anaesthesia as well as analgesics during the post-operative recovery period.

We will use models of traumatic brain injury (TBI) that focus on small regions of the brain. We can use mechanical controlled contusion, aspiration lesions or other methods to induce TBI. Our techniques are chosen because their location and size are easy to control. Thus, these models do not result in observable changes in movement, feeding and other functions, and typically do not result in weight loss or other lasting harm.

Aging mice will be used to understand how plasticity and repair change in old age. We will use an extensive number and variety of humane endpoints to ensure that animals do not experience excessive or lasting discomfort.

Our two-photon and three-photon imaging methods are well tolerated and in and of themselves do not cause pain or lasting harm. Imaging is typically carried out through glass windows affixed to the skull and involve animals that have been habituated to head-fixation. Animals habituate readily to head- fixation

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 typically tolerate water restriction with no adverse effects. Body weight provides a robust measure of health before any more serious signs of dehydration are observed, such as hunched posture or piloerection. This allows us to provide supplementary water as necessary to prevent adverse effects.

Maternal separation and early weaning (MSEW) one of the behavioural protocols used in protocol 3 is designed to mimic postpartum depression (PPD) but does not cause adverse effects for the animals that are more than mild and transient.

Why can’t you use animals that are less sentient?

Animals less sentient than mice and rats such as invertebrates lack cerebral cortices and are deficient in their behavioural repertoire in comparison to humans. Invertebrates do not exhibit complex motor behaviour and learning to the degree that mammals do. Invertebrates and other “lower organisms” however exhibit superior brain plasticity and repair then mammals. Thus, they are not appropriate for research mechanisms to increase plasticity – they are already quite plastic.
We have chosen post-natal and adult life stages to mostly focus on since embryos do not typically experience neurodegeneration and psychiatric illness, as far as we know. Similarly anaesthetised mice or rats do not exhibit complex motor behaviour and learning. We will also use a small number of embryonic animals to determine if neuroplasticity is influenced by events in utero. These events (e.g. inflammation) are major risk factors for psychiatric disease.

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 animals on a daily basis. For mice previously having undergone surgery we consult with the NVS and provide additional measures for any mice with specific needs. This may include moist food to ensure weight maintenance and medication to relieve pain or clean and heal wounds. For animals 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.

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 practise is followed for refinement. We will also follow NC3R’s published guidelines on rodent head fixation and fluid/food control.
We will also follow NC3R’s published guidelines on rodent head fixation and fluid/food control.

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 will continue to adopt any new approaches that allow us to improve the three Rs. We will also engage with ongoing institutional national 3R's efforts including establishment welfare meetings and 3R’s days interacting with the Named Information Officer and signing up to the NC3R’s newsletter.

56. Mechanistic Models of Immunology

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

Immunology, Inflammation, Therapy, Autoimmunity

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?

The overall aim of this project is to test novel therapeutics that will allow patients suffering from immunological diseases to receive better treatments which will achieve long-term remission without the need for continuous medication. A sub-aim is to discover novel biology in the area of immune-driven diseases to help us meet the overall 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?

Diseases of the immune system (such as auto-immune diseases) represent a significant burden on the lives of patients, and also on the healthcare system.

There are no cures for chronic immune-mediated inflammatory diseases such as rheumatoid arthritis (RA), diseases of the spondyloarthritis family (SpA), connective tissue diseases, dermatological inflammatory conditions (such as psoriasis and atopic dermatitis), inflammatory bowel disease (IBD), asthma and autoimmune neurological diseases such as multiple sclerosis.

Patients currently rely on life-long therapies to control symptoms, but often these therapies do not address the problems with the immune system that drive the disease.

New, transformative therapies are required to fulfil the unmet patient need, and to address mechanisms of disease that do not just control symptoms but move towards a genuine cure for patients.

Through this program of work, we expect to support the development of one or more novel therapeutics (e.g. novel small molecules, antibodies or protein therapies) into clinical trials and therefore benefit patients living with immune-cell diseases. Due to the drug discovery process this is likely to be a medium to long term benefit (5 – 10 years).

In the shorter term, we expect to run in vivo models that can be incorporated into a rational screening cascade to support projects as they progress towards the clinic. These models may benefit multiple projects within our company. They can also help unravel novel biology and drive understanding of the mechanisms of action of novel therapeutics. This can lead to better positioning of the therapeutic in the clinic increasing the impact of treatment on certain patient populations. Assessing the underlying mechanisms in these models may also aid research/project teams to identify new targets, with the aim of generating novel therapeutics, and new scientific understanding in the field.

Data on models as well as novel therapeutics are often shared at conferences in the form of posters and as oral communications, or published in scientific journals. We will also collaborate with both industry and academia and share methods, protocols and best practices from our experience in this area of research.

What outputs do you think you will see at the end of this project?

The fundamental output of this licence will hopefully be therapeutics for immunological diseases. However, in the shorter term, outputs will be measured in terms of candidate molecules, refinements of the protocols within this project, and scientific publications.

Who or what will benefit from these outputs, and how?

The ultimate beneficiaries of this licence will be patients suffering from diseases mediated by the immune system. However, in the shorter term there will be benefits to the scientific community as we will strive to publish and publicise all refinements, methods, protocols, and best practices developed during the course of this project. These short term benefits

may be increased understanding of the processes auto-immunity, and the roles of various ligands and receptors in this process, improved scientific protocols, refinement of in vivo models, or the identification of novel targets in immune diseases.

How will you look to maximise the outputs of this work?

We will seek to collaborate with leading academic institutions to ensure our methods and protocols are always utilising the highest standard of welfare and scientific rigor.

Papers, posters, and talks will be prepared and presented at research conferences or published in appropriate leading research journals.

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.

Mice are the mammalian species of lowest neurophysiological sensitivity in which these immunology models have been developed. Mice also have an immune system broadly similar to that of humans, allowing the study of immune cell interactions. It is not possible to run models of this nature in neonatal animals as their immune systems differ in important ways, and therefore adult animals are required.

Typically, what will be done to an animal used in your project?

As the project utilises a number of different models of disease, there is not one typical experience an animal will undergo. However, the general steps of the protocols of the licence would be as follows:

1. Immune challenge – this may be immunisation, cell transfer, or this could be through introduction of a damaging agent which may induce a clinical disease (such as in the induced colitis model). Some animals may be sensitised and then challenged with the same agent on up to three occasions, and their response to this challenge measured.

2. Disease monitoring - for some models this will consist of weighing and disease scoring several times per week. For other models this will encompass sampling of blood, urine, and / or faeces to determine immune cell populations, drug concentrations, or other parameters such as tracking specific cell populations or assessing vascular permeability.

3. Application of a therapeutic - This may be by intraperitoneal (IP), intravenous (IV), or subcutaneous (SC) injection, or whichever route is most appropriate for the compound

or molecule being tested. Animals may be dosed one time only or on multiple occasions (likely not more than 1x daily).

4. Animals will be terminated at the end of the procedure by a method of humane killing. Usually, post mortem tissues will be collected for analysis of immune cell populations in primary and secondary lymphoid tissues, and in organs of interest. Samples may also be taken to assess disease pathology.

What are the expected impacts and/or adverse effects for the animals during your project?

Often, mice on this project will only experience the very mild and transient suffering of an injection with a small-bore needle. The majority of the immune challenges in this project are not expected to induce any clinical symptoms.

In some models, the animals will undergo procedures that may involve injections of damaging agents or antigens, after which they may experience clinical signs of disease such as swollen ears, swollen paws, or irritable bowel symptoms.

Animals may undergo procedures that mimic autoimmune diseases such as Graft vs Host Disease. These mice may undergo an inflammatory response. These aspects of disease may lead to weight loss and a general loss of 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)?

70% mild

30% moderate

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Much of our work is done using cell culture systems. However, the immune system is complex and multi-faceted - at this stage, there are no in vitro models that allow sufficient capture of immunological mechanisms to replace animal models fully.

To model the pharmacokinetics and pharmacodynamics (PK/PD) of novel therapies, we can only use animals with a functional immune system, tissues, blood vessels, lymphatic systems, and all other components that affect the PK/PD of drugs.

The modelling of diseases is becoming more possible in vitro, but we are still a long way from being able to model all aspects of autoimmunity in a way that captures the interplay seen in the human condition.

Autoimmunity and other diseases with an immunological component consist of a complex interplay between many cell types which currently can't be recreated in cell culture.

Which non-animal alternatives did you consider for use in this project?

Various models of disease mechanisms are present in cell culture conditions, and where these meet the experimental goals, they will be used. They were considered for the course of the project.

These include biophysical assays to determine the appropriate molecular interactions of our novel molecules, reporter cell assays to determine if our therapeutics are engaging the correct signalling pathways at a basic level, and primary cell assays to model the response of immune cells in a particular setting. Organ-on-a-chip systems are also an exciting new development that may in the future allow researchers to move away from animal models - and we considered these here.

We also considered precision cut tissue slices – but while these are becoming a mainstay of research in other areas (such as fibrosis) their utility in immunology is still limited.

Why were they not suitable?

Cell culture models (both 2D and 3D) and reporter assays are useful for assessing individual mechanisms and modalities, but the combination of these mechanisms is the driver of immunological diseases. It is also not currently possible to predict whether the magnitude of effects seen in vitro will translate into a clinically meaningful impact on disease in the absence of in vivo models.

Currently, only in vivo models allow for all aspects of disease to be modelled together.

Typically the in vitro assays mentioned will be used prior to any testing in vivo, to ensure only the most promising therapeutic candidates are tested in animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Animal estimates here are based on experience over previous years (working under a separate project), and forecasting of the number of current and future projects which will progress to in vivo testing.

Typical group sizes would be 8-10 animals.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have discussed group sizes for various experimental protocols with statisticians, and have also used online tools such as the NC3R's Design Assistant to aid us in reduction.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

To ensure the fewest number of animals are used, only the most effective drugs that have been pre- screened for activity in vitro will be examined in animals.

Our experience with the experimental protocols will be applied to ensure appropriate group sizes are used to identify statistically significant differences between groups, whilst minimising the numbers of animals undergoing the protocol. Group sizes are constantly reviewed and experts in statistics and modelling are consulted to ensure the minimum numbers of animals are used.

Pilot studies may be performed using novel therapeutics in the first instance, and more in-depth studies performed once these molecules have shown promise in these initial pilot studies.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

This project encompasses the use of both basic mechanistic models of immune cell activity, and more complex disease-relevant models.

Where possible, scientific questions will be answered using the model which causes the least amount of distress to the animals.

To able to study human immunological diseases it is essential that we first create our disease model. This means animals will exhibit some disease phenotypes. All protocols and monitoring within this project are designed to keep suffering to a minimum.

Why can’t you use animals that are less sentient?

Fish are not a suitable species for this work as they lack an immune system that is representative of that of humans.

Mice are the mammalian species of lowest neurophysiological sensitivity in which these models have been developed.

Neonatal animals generally have a lesser developed immune system, and therefore are not always suitable for research.

The duration of the models precludes the use of terminally anaesthetised animals, as many of these models are run for 7 days or longer.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Monitoring of the animals and welfare scoring sheets will be adapted and refined over the course of this project.

As new techniques or models become available or established, the procedures containing within this project will be reviewed and updated periodically.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We are and will continue to review relevant publications both available via NC3Rs and LASA, and academic literature, to ensure our models run in the most refined way.

In house experts will be consulted for experimental design input, and we make good use of the resources such as the Experimental Design Assistant offered by the NC3Rs Resource Library to ensure experimental design is optimal.

All publications will adhere to the ARRIVE guidelines

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Members of our in vivo pharmacology team will routinely attend conferences such as LASA, FELASA, and conferences organised by the RSPCA to discuss advances in 3Rs and to bring back learnings that can be implemented into the project. These may be through simple changes, or through periodic amendments to the licence to better reflect best practice. The team will also remain networked with other researchers, Named Animal Care and Welfare Officers, and technicians within the establishment.

57. Neuronal control of the heart

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

autonomic nervous system, heart failure, myocardial infarction, arrhythmia

Animal types	Life stages
Mice	adult, juvenile
Rats	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to better understand the pathophysiology of molecules such as neuropeptide-Y (neuromodulators) and how they influence the neuronal control of the heart and it's blood supply and the mechanisms by which they can contribute to worsening heart function and dangerous heart rhythms following heart damage.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

We have identified differences in key signalling molecules in neuronal tissue from normal animals and those with cardiac disease. One such molecule is neuropeptide-Y released by a group of nerves that supply the heart and it's blood supply, and preliminary work has identified some of the pathways by which it may be acting. Our translational studies in patients suggest that high circulating levels of neuropeptide-Y are associated with a poor prognosis following heart attacks and during heart failure. We hope that understanding how the heart, blood vessels and nerves communicate will lead to the development of new biomarkers to identify patients at high risk following heart attacks and during heart failure and help us identify potential new targets by which neuropeptide-Y and other molecules may be acting against which drugs may be developed.

What outputs do you think you will see at the end of this project?

We aim to publish new findings in peer reviewed journals that improve our understanding of the disease processes around heart attacks, heart failure and arrhythmia and the role the nervous system plays in the pathophysiology. Outputs may include new signalling pathways, key molecular players, and potential pharmacological targets.

Who or what will benefit from these outputs, and how?

This work is a continuation of an avenue of research that has already made important advances in the field which have been published in high impact journals thereby benefiting the wider scientific community. In the short term we hope that further validating our findings in translational human studies including work on neuropeptide Y as a biomarker will benefit patients. The project also has the potential to identify other new biomarker molecules, and with a better understanding of their signalling pathways in both animal models and human tissue, we can then start to build an evidence base for the effectiveness of targeting their pathways which Pharma could subsequently exploit in the longer term.

How will you look to maximise the outputs of this work?

We collaborate with several research groups within the UK and the USA in producing research grants and then work together on the science to produce high impact publications. We help to promote our findings and receive feedback by presenting work at international conferences. After peer review and acceptance for publication, we publicise our work and engage with the wider public through press releases via our institution and funding bodies to help disseminate the findings widely.

Species and numbers of animals expected to be used

- Mice: 1000
- Rats: 1500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use rats for our work in hypertension as they are models in which hypertension naturally occurs and other models are available where hypertension is induced. These mirror the human condition well. Mice will be used as they are the species most likely to be genetically modified to mimic human cardiac disorders. As problems in the nerves innervating the heart arise before the development of high blood pressure and other cardiovascular disease, our project will use both juvenile and adult animals before the onset of the condition.

Typically, what will be done to an animal used in your project?

Mouse models with no more than a mild cardiac dysfunction, will be sourced from other projects, which have authority to supply them. We will then maintain them on this project so that we can study the cardiac function at various time points in the animal's life.

Rat models of hypertension will also be sourced from commercial suppliers and other projects with authority to supply these models. These animals will be maintained up to a maximum of 12 months which is before the risk of heart failure occurs. Typically, we will evaluate their heart function between 4 and 20 weeks of age.

To enable us to investigate the animal's cardiac status, animals will either be humanely killed and then tissues harvested postmortem, or tissues will be collected under terminal anaesthesia with subsequent humane killing.

What are the expected impacts and/or adverse effects for the animals during your project?

Spontaneously hypertensive rats will develop high blood pressure and enlargement of the heart with advancing age. They will be kept up to a maximum of 12 months of age when there is minimal risk of heart failure or stroke. Typically, we use this model by 5 months of age before there are any symptoms.

We do not expect our mice models to experience significant symptoms either. As we do not plan to breed these animals but source them from other projects, we will receive full details of their health that may have an impact of a mild severity that we need to monitor for.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Rats: sub-threshold 90% mild 10%

Mice: sub-threshold 70% mild 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?

There is no replacement alternative for studying the complex organisation of neurons that exists in and around the heart and how they respond to injury. It is also not possible to study whole organ behaviour such as the generation of arrhythmias or remodelling where structural anatomical features within the organ are key.

Which non-animal alternatives did you consider for use in this project?

We routinely use human skin cells that have been reprogrammed and re-engineered to become heart and neuronal cells (known as induced pluripotent stem cells or iPSCs). These cells can then be studied in isolation, heart cells grown in layers on their own or with neuronal cells. This has greatly reduced the number of animals we use for simple cellular work. We also do translational work in patients being treated for myocardial infarction and heart failure although studies are limited to imaging, limited tissue samples, blood sampling and some invasive measure of haemodynamics.

Why were they not suitable?

iPSC are often developmentally immature and do not always function the same as when cells are isolated directly from organs in humans and animals. In addition, it is not possible to recreate whole organ structure and behaviour. This is particularly important when studying blood flow or dangerous heart rhythms that arise from specific parts of the heart, or how the organ changes in response to injury or high blood pressure.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have taken statistical advice with regards to our calculations using typical variations from our extensive experience with the techniques we perform to calculate a minimum number of animals to be used whilst ensuring that the results are statistically significant. Sample sizes for our experiments are estimated based on this experience and calculations which typically show that a group sizes of 10 (with a typical technical failure rate of <5% in completing all aspects of a full tissue dissection/experimental protocol to obtain a full data set) is required to achieve the quality of results we need. We have also used our annual return of procedures data from previous projects to estimate the number of animals that we will need, although as our cellular work moves more to iPSCs, then it may well be fewer.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Through years of research experience, we are highly familiar with the use of appropriate statistical methods. Furthermore, power calculations are routinely performed when experiments are designed and incorporate potential technical failure rates which have been refined over the years to be as small as possible. This includes the optimisation of approaches to dissection, concentrations and compositions of the solutions used to grow cells and keep tissues alive, and design and running of equipment to take reliable measurements. The minimum numbers of animals required have been carefully reviewed by the funding agencies and incorporate our own data as well as that published by renowned research groups in the field. These are continually reviewed as projects progress. We conduct our experiments to 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?

We will use previous published data and our own pilot studies to help generate appropriate power calculations for experiments that will be re-evaluated as the work progresses. Where possible computer modelling can be also used to make predictions regarding potential effect size. After humane killing, we will ensure that tissue will be harvested for potential later use in other protocols if suitable, to maximise the data that could be obtained from every animal.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The rodent models chosen are the smallest possible with regards to the compromise between having similar properties in the function of their hearts and nerves to humans. The best model for hypertension is the spontaneously hypertensive rat which we have years of experience with regarding measurements and experiments. Other models of disease will also be used but will be imported from a named supplier or another project. Mouse is the best model for specific genetic mutations associated with the disease we study. All experiments will be carried out under terminal anaesthesia or after humane killing.

Why can’t you use animals that are less sentient?

Non-mammalian species have structurally different hearts and cardiovascular systems with different neuronal innervation and electrophysiological behaviours which limit our ability to make valid conclusions from their use.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We do not propose to perform any procedures on rats or mice that are not postmortem or under terminal anaesthesia. We will liaise with the named animal care and welfare officer to ensure any rats or genetically modified mice are monitored appropriately.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will ensure that we follow the general guidance in relation to refinement (such as www.nc3rs.org.uk, https://norecopa.no, https://www.lasa.co.uk) and make use of our Named Information Officer. In addition, we will follow best practice technical guidance with relation to specific experiments and their reporting as this evolves.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay up to date with published advances in our research field that demonstrate refinement and replacement of relevant experimental techniques that will lead to a reduction in animal usage. In addition, we will attend internal 3R’s meetings, stay in communication and engage with our Named Information Officer as well as use information provided through websites such as www.nc3rs.org.uk and https://science.rspca.org.uk

58. Pharmacological evaluation of imaging agents and development of imaging applications and methods

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

Imaging agents, Pharmacokinetics, Biodistribution, Metabolism, Imaging methods

Animal types	Life stages
	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 evaluate the pharmacological properties of imaging agents,
- to develop imaging methods and
- to investigate applications of the imaging agents.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 clinic, imaging studies (Computerised Tomography (CT), Magnetic Resonance Imaging (MRI), Positron Emission Tomography (PET) etc.) provide the means to visualise and quantify the status of specific molecular targets in living subjects in a minimally invasive manner. Such imaging studies could be for the purpose of research into understanding a disease, diagnosing a disease, assessing the stage of the disease, for treatment selection, evaluating treatment effects, patient selection and grouping for clinical trials etc. As new molecular targets involved in diseases are discovered, the imaging agents that are required for imaging them need to be developed and evaluated in a manner similar to the drug development process, typically involving steps such as

1. Drug design

Lead compound identification

Chemical modification of the lead compound and generation of a compound library
Screening the compound library to select candidate compounds for further evaluation
Production of the candidate imaging agent

2. **Biological evaluation**

1. In vitro (not in a living organism) evaluation of the candidate imaging agent

2. **Preclinical in vivo (in a living organism) evaluation of pharmacological properties (eg. does it enter the brain?, does it break apart?) of the candidate imaging agent, development of imaging methods and investigation of applications of the imaging agent**

3. Preclinical in vivo evaluation in disease models

4. Toxicological evaluation

3. Translation to first-in-human studies

In this process, this PPL (project licence) aims to address the step 2.2. The overall aim of this PPL is to investigate the pharmacological properties of imaging agents that are often independent of disease conditions and to develop associated imaging methods to be able to use the imaging agents in practice.

What outputs do you think you will see at the end of this project?

This PPL will be the first step in in vivo evaluation of imaging agent candidates.

1. Agents with properties that make them suitable for use as imaging agents will be identified from potential candidates. Agents that are not suitable will not proceed to evaluation in disease models with higher welfare concerns.

2. Imaging methods that can be used for evaluation of imaging agents or disease processes will be developed.

3. Questions raised about properties of imaging agents (e.g. Does the imaging agent bind to any other targets?) or imaging methodology (e.g. Can the imaging agent be repurposed for imaging other biological functions?) will be answered.

4. Publication in peer reviewed scientific journals, dissemination of findings, including unsuccessful approaches or non-significant data via open access and through platforms such as F1000 Research

Once an imaging agent is developed with the appropriate characteristics (2-4 years of development), it will be moved on to disease specific PPLs with relevant disease models for further evaluation.

Who or what will benefit from these outputs, and how?

In the short term, better understanding will be developed regarding the relationship between the chemical structures of the imaging agents and their biological activity. Novel imaging methods will be developed in order to effectively use imaging agents to study changes that happen in a given disease.

In the medium term, imaging agents developed and used in preclinical disease models will aid the understanding of disease processes and play a part in the evaluation of potential drug treatments that modify the imaging targets.

These will be circulated in the public domain in the scientific community, guiding future direction in imaging agent development. Thus, in the short to medium term, the beneficiaries are researchers in the imaging and drug development community.

In the long term, studies in this PPL will contribute to development of minimally invasive imaging agents and methods for diagnosis of diseases, choosing the right treatment for the right patient or for development of drug treatments.

For example, imaging agents that bind to specific toxic misfolded proteins (such as tau, alpha synuclein, Huntingtin etc.) deposited in the brain in various forms of dementia will help to diagnose which type of dementia a patient has. Such toxic proteins are formed years before the clinical symptoms develop. When new drugs for removing these proteins are being developed it will help to monitor whether the treatment decreases the levels of the toxic proteins during clinical trials and in practice.

Choosing the right treatment for the right patient is the aim of the project where imaging agents are being developed for brain stem cells called oligodendrocyte precursor cells

(OPCs). These cells can develop into the cells that form the myelin sheath which protects neurons. In multiple sclerosis the myelin sheath is damaged and drugs are being developed to convert the stem cells into myelin forming cells. However, these drugs will only be useful for those patients that already have the stem cells. The imaging agent will help to identify which patients are likely to benefit from the drug treatment.

Inflammation is involved in several diseases. However, whether it is the cause or result of the disease is often not clear. Imaging agents that identify markers of various stages of inflammation, especially in combination with imaging for other aspects of the disease will help to understand the disease process better and hence to develop better treatments in the future.

Thus, patients are the eventual beneficiaries.

How will you look to maximise the outputs of this work?

Publication in peer reviewed scientific journals or local, national or international conferences, dissemination of findings, including unsuccessful approaches or non-significant data via open access and through platforms such as F1000 Research.

Collaborations with laboratories and institutions with expertise in different scientific areas to include chemistry, radiochemistry, chemical engineering, pharmacy, physics, clinical imaging and clinical and veterinary medicine have been established to carry out the proposed work.

Species and numbers of animals expected to be used

- Mice: 220
- Rats: 320

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 wildtype (normal, healthy, without a disease causing genetic change) mice and rats will be utilised.

While some of the preliminary work can be done using in vitro systems, testing the pharmacological properties in healthy, non-diseased rodents is the mainstay of preclinical development of imaging agents and methods. They allow us to demonstrate that the imaging agents and methods have potential for further application in humans as they are a good model for the physiological processes in humans.

They are therefore suitable for assessment of the pharmacological properties of the imaging agents and allows for fast failure of imperfect agents before further application in animal models of disease. For example, PET imaging will tell us in three healthy animal scans if an imaging agent for brain does not pass the blood-brain barrier, thus, it will not be progressed into a disease model. The biological insight gained from such studies in healthy wildtype animals will instead be used to modify the chemical structure of the imaging agent for developing the next series of imaging agent candidates.

Typically, what will be done to an animal used in your project?

The procedures carried out on these animals are standardised, generally accepted and will be conducted abiding to humane treatment conditions of animals involved.

Most studies (Protocol 1) will be non-recovery studies where all procedures are performed in anaesthetised (unconcious) animals. They are administered candidate imaging agents (such as small amounts of radioactively labelled small molecules, peptides or proteins to larger structures such as synthetic nanoparticles, radiolabelled cells or other contrast agents) typically via intravenous injection but various routes such as intraperitoneal, subcutaneous or intramuscular may be used instead. Fur may be shaved for surgical placing of cannulas in blood vessels. Agents that interfere with the imaging agent (such as drugs, unlabelled candidate imaging agents, other competing agents) may be administered similarly by various routes to the animals depending on the properties of the individual agent. The animals will either undergo imaging procedures or tissues/blood samples are collected for analysis. Where compounds of unknown properties are administered for the first time, it will be administered in non-recovery studies.

The imaging procedures are carried out under anaesthesia and can last from a few minutes (a 6 minute CT) to a few hours (typical PET-CT or MRI scan of 1-2 hours or rarely up to 4 hours when combining multiple imaging modalities or imaging with radiotracers with longer half-lives). Some studies will involve imaging (eg., CT, MRI) without administration of imaging agents (Protocol 3). At the end of these studies the animals are killed humanely without waking up from their anaesthesia.

Some studies will involve preparing the awake animals for the scan (Protocol 2 and 4). This could be food withdrawal or different feed with or without substances that may interfere with the imaging or pre- treatment via routes such as drink or food, oral administration, or injection routes such as intravenous, intraperitoneal, subcutaneous or intramuscular with imaging agents or agents that interfere with the imaging agent or process depending on the properties of the individual agent. This will be followed by imaging as above or tissue/blood sampling under anaesthesia or urine / faeces collected for analysis while housed in isolation. Where animals are housed in isolation, it will usually be for 1-2 hours or if longer, they will be culled at the end of the isolation period.

A small number of studies (Protocol 2 and 4) will involve multiple scans, on up to 5 occasions, over several days and may involve preparation of animals for scans or sample collections as above (food withdrawal, different feed, administration of interfering

substances or imaging agents). Where animals are recovered from anaesthesia, warming boxes will be used to aid in recovery.

At the end of the experiments animals will be killed by a humane method such as overdose of anaesthetic and organs and tissues may be collected for further experiments. Tissues may be frozen and stored for use in future studies.

What are the expected impacts and/or adverse effects for the animals during your project?

Most procedures will be carried out in unconscious, anaesthetised animals and with careful monitoring there should be no side effects to the animals except for transient discomfort from being anaesthetised.

Preparing the awake animals for studies may cause mild discomfort from being handled for administration of substances via injections (transient discomfort), hunger from short term withdrawal of feed (less than 12 hours, typically 4 hours) or anxiety from rare occurrences of individual housing (1-2 hours if returning to home cage or very rarely up to 24 hours on a single occasion at the end which they will be humanely culled). Some animals will be anaesthetised for imaging on up to 5 occasions which might result in anxiety leading upto the next anaestheisa and during the recovery from anaesthesia.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice

Non-recovery: 64%

Mild: 36%

Moderate: 0%

Severe: 0%

Rats

Non-recovery: 75%

Mild: 25%

Moderate: 0%

Severe: 0%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

It is necessary to conduct studies on animals as the complex interactions between organ systems that may affect the behaviour of the imaging agents in the body cannot be replicated by in vitro studies or computer models.

Which non-animal alternatives did you consider for use in this project?

We currently use several tests, depending on the kind of imaging agent, before proceeding to animal studies as follows.

1. Computer modelling will be used where possible to narrow down the library of candidate compounds to those with the best properties.

2. The ability of the imaging agent to penetrate biological membranes which consists of oil-like and water-like parts will be tentatively evaluated by the octanol-buffer partition method. In this method the imaging agent is added to a mixture of an oil-like (octanol) and water-like (buffer) substances and the proportion of the imaging agent distributing into each part evaluated.

3. In vitro binding studies in archived human or animal tissues or cells etc. will be performed where possible to rank the candidate imaging agents. Only agents with good binding properties (often in nanomolar range) will proceed further.

4. The Imaging agent formulation will be tested for stability for example, on the benchtop for typical storage durations, in animal or human plasma or in commercially obtained liver extracts for typical scan durations.

5. Imaging phantoms (objects that stand in for animals) or cadavers will be utilized where possible in order to minimise the number of animals required for developing imaging methods.

Why were they not suitable?

In vitro and computational methods are not fully representative of the biological characteristics of living organisms. These non-animal alternative methods can only provide preliminary and partial information on the pharmacological properties and target selectivity of the imaging agents to allow preliminary screening and selection of the best agents for further evaluation in animals. Only animal models can reproduce the complex interactions between organ systems that may affect the behaviour of the imaging agents.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 for each experiment are based on statistical consultation, references from published scientific papers and our previous research experience and published track record in conducting studies on small animal imaging.

Initial studies for the evaluation of novel candidate imaging agents are exploratory and aims to answer questions such as “Which organs do the imaging agent distribute to and in what time frame?”, “Does the imaging agent break apart into radiometabolites after administration into an animal?”. Such qualitative studies typically require about 6 animals per imaging agent and will serve as pilot studies for further evaluation of suitable agents.

When there is a need to statistically compare groups, information obtained from the pilot studies will be utilised to estimate the required number of animals. About 10 animals per group is typical in such imaging studies.

The total number of animals required over the 5 year period was estimated based on the typical number of researchers developing imaging agents within the group and the number of imaging agents that are likely to be evaluated within this time period. Depending on the success of the projects, this number may vary, necessitating an amendment.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Experiments will be carefully planned and controlled to reduce variability. All experiments will be designed in consultation with radiochemists, biologists, imaging scientists and animal facility staff via study plans. Imaging data analysis plan will be developed under the guidance of trained imaging scientists and statisticians consulted where required. Endpoints for which the experiments are powered are discussed and defined before each experimental plan.

In most experiments, the animal is imaged over 1-2 hours, providing information from multiple time points within that time which will reduce the overall number of animals necessary for the research. For example, a group of animals are imaged dynamically from 0 min to 2 hours in order to obtain the time dependent distribution of imaging agents in to various tissues rather than killing different groups of animals at various time points within those 2 hours.

Where possible, blood samples for determination of plasma levels of the imaging agent and radiometabolite levels will be obtained from the same animals that are being imaged. Obtaining all this information from individual animals will contribute to reducing the variability in the data and ultimately leading to a reduction in the number of animals required. To facilitate this, rats are chosen over mice for studies requiring blood sampling. In addition, tissues may be harvested from the animals after the last imaging time point for archival and further in vitro evaluation as required.

For studies where multiple groups are compared, animals will be randomised to the control and treatment groups. Image analysis is performed using automated software which provides opportunity for blinding.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Computer modelling and in vitro studies on human or rodent tissues will be used to decide whether the imaging agents can proceed on to animal testing.

Where possible tissues obtained after the animals are killed will be utilised to generate pilot data for future experiments. Being a part of a large biomedical research organisation, such tissue may be obtained from other groups within the organisation via internal mailing lists.

Pilot exploratory studies will first be conducted on small groups of terminally anaesthetised animals to quickly identify unsuitable candidate imaging agents that are then removed from further evaluation as well as select the best imaging agent for further evaluation. Such studies are additionally used to determine the number of animals required to obtain robust data in larger 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.

Wildtype rats and mice will be used in this project. In most cases the animals will be terminally anaesthetised, causing the least suffering.

Dedicated imaging equipment (for example PET, CT, MRI etc.) for use in mice and rats will be utilised in this study. These scanners are designed to closely mimic state of the art

clinical scanners and have been developed with imaging of rodents in mind as these models are well recognised to be the ideal preclinical model for this type of setting.

Most studies will involve imaging or blood / tissue sampling in terminally anaesthetised animals. Some studies will involve preparing animals for the scans while awake (for example, by administration of substances, food restriction) and then performing the scans in anaesthetised animals. In some studies, the anaesthetised animals will be woken up after the scan and the scanning procedure repeated on other occasions. These methods cause the least suffering to obtain this amount of data.

Why can’t you use animals that are less sentient?

Rodents are widely recognised as the least sentient species to carry out research of this type. Pharmacological properties are typically evaluated in adult rodents as their organ systems are mature and representative of human adults. The scanners used are miniatures of human scanners and specifically designed for rodents. The inherent resolution of the imaging techniques (for example, about 1mm for PET) necessitates the use of large enough animals such as mice or rats to be able to accurately quantify the imaging data. Rodents allow for the administration of imaging agents in quantities that can be detected by the scanners as well as to sample blood in sufficient quantities (in the case of rats) for establishing image quantification methods.

Where possible, studies will be performed in terminally anaesthetised animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Scanning protocols can be refined (injected doses, scanning duration, blood and tissue sampling and analysis protocols etc.) based on information gained from pilot studies.

The doses and routes of administration of interfering agents/ unlabelled drugs will be chosen based on established procedure, literature or prior pilot studies to obtain scientifically valid information while not producing adverse effects beyond transient discomfort due to the injections.

During the scans, the anaesthetised animals are placed on scanner beds that are warmed and the animals are physiologically monitored, typically rectal temperature and breathing rate.

In case of longitudinal studies, after each occasion of scanning, animals will be kept in an individual warm environment with access to food and water to recover from the anaesthesia. Anaesthesia will not be re-induced until the animals have recovered from the previous instance of anaesthesia. Where longer scanning durations (2-4h) are required, fluid replacement (warm saline/dextrose) may be administered.

Animal suffering will be minimised by careful observation of the animals undergoing procedures. Guidelines for the assessment of clinical signs will be strictly followed, and

experiment on the particular animal or cohort will be immediately terminated and the animal will be humanely killed upon observation of clinical signs.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Guidance and best practise will be followed, for example Refining procedures for the administration of substances.

https://journals.sagepub.com/doi/pdf/10.1258/0023677011911345 Research Animal Training: https://researchanimaltraining.com/

PREPARE guidelines and checklists will be consulted. https://norecopa.no/about-norecopa NC3Rs website https://www.nc3rs.org.uk/.

Focus will be given to the updated ARRIVE guidelines https://doi.org/10.1371/journal.pbio.3000410 updated in 2020 to design experiments that can be properly executed and reported.

ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments) https://arriveguidelines.org/ Other resources from these websites will be consulted.

(LASA) Laboratory Animal Science Association

(AALAS) American Association for Laboratory Animals Science

(FELASA) Federation of European Laboratory Animal Science Associations (ICLAS) International Council for Laboratory Animal Sciences

(InterNICHE) International Network for Humane Education

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

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The organisation has extensive documentation regarding 3Rs including links to publications, portals and websites with information pertinent to all commonly used laboratory animals. This information is continuously updated and distributed via mailing lists. Staff involved in animal handling and experiments undergo rigorous and continued training on these issues and have all these resources available.

59. Modelling and therapeutics for neurodegeneration

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Gene therapy, Neurodegeneration, Disease, Treatment

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?

New scientific methodologies, such as gene therapy, have been developed which could enable us to replace or repair the DNA mistakes. Our objective is to understand how these mistakes cause neuronal injury and to develop treatment for diseases on the brain and spinal cord.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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), e.g. childhood and adult neurological (nervous system) disorders, have huge personal and societal impact. These diseases are often very severe, genetically driven illnesses with high morbidity and mortality that place a large burden on patients, their families and healthcare systems. These conditions, for which there is a clear unmet need for effective therapies, are the diseases upon which our proposed project will focus.

Curative therapies for rare and orphan diseases have been a long-held, pertinent unmet need for both patients and many in the biomedical research and development arena. Though these diseases are relatively rare, the costs of medicines are high, even when they provide only marginal benefit.

Development of safe, efficacious (successful in producing desired effect) and cost-effective therapies for rare diseases represents a major challenge because of the nature of these conditions.

What outputs do you think you will see at the end of this project?

Our ultimate aim is to translate our discoveries from lab to human clinical trials. For instance, a gene replacement therapy we recently developed under our active PPL is progressing towards first in man clinical trials. We successfully completed proof-of-concept and safety studies and currently preparing a new Investigation Drug (IND) for submission to the USA Food and Drug Administration (FDA) in next 3- 6 months to request approval to treat patients. In addition, 2 of our gene therapy products are being commercialised by the recently established University spin out companies. We therefore anticipate similar impactful outputs from our future pre-clinical studies.

Other outputs includes new Intellectual Property (IP) (patent filling) and high profile publications enabling sharing of our findings with scientific community. Our pre-clinical studies and track record in the field enabled to lead on highly competitive awards (e.g. EU Innovative Medicines Initiative, MRC/LifeArc) to initiate new innovation projects in the UK.

We are currently generating 3D models for neurodegeneration under current programmes.

Who or what will benefit from these outputs, and how?

The expected benefits of the work are:

1. We will have a greater understanding of the molecular and cellular mechanisms of neuronal injury in neurodegenerative diseases: e.g. MND and HSP.

2. Therapeutic development: exciting data emerged already from our previous work. We expect our therapeutics to progress towards clinical development. Further gene therapeutic strategies are currently at pre-clinical stage.

Early time point: The know-how and expertise developed by the proposed work will be shared with scientific community via publications and conferences.

Medium time point: generate i) better understanding disease mechanisms and ii) strong proof-of- concept for therapy to serve as basis for clinical development. Examples of strong proof-of-concepts from our work include survival motor neuron (SMN) replacement strategy for spinal muscular atrophy. In pioneering first proof-of-concepts, our team demonstrated that SMN gene replacement restored the SMN protein in skin cells from children with SMA and, using an AAV9 viral vector to deliver the therapy, prolonged survival in mouse models of SMA, an approach that received Orphan Drug Designation from European Medicine Agency (EMA). Clinical trials conducted in the USA showed that AAV9-SMN gene therapy led to babies achieving developmental motor milestones where they previously would have declined. Zolgensma (AAV9-SMN) is now delivered as a life-saving therapy to patients globally.

Late time point: i) clinical trials to treat patients affected the diseases at the heart of this PPL; ii) Creation of start up companies to help progress these therapies to market which would have economical benefits. Our previous work led to the establishment of two start up companies.

The work will be disseminated by i) publications in international scientific journals; ii) presentation at national and international conferences; iii) annual open day events accessible for families and patients;

iv) Public Patients Involvement and Engagement (PPIE) activities under current programmes; v) 3Rs events and committees attended by the team and the applicant.

How will you look to maximise the outputs of this work?

We aim to maximise the outputs of our project through various channels:

1) Collaboration with investigators in UK and worldwide institutions.

2) Present our findings at national and international conferences

3) Report both positive and negative (unsuccessful) data and methodologies in established scientific journals

4) Share our knowledge at funding Committee/Boards: e.g. The applicant has been a Panel member of the CRACK IT Challenge - Challenge Panel: NC3Rs – National Centre for the Replacement, Refinement and Reduction of Animals in Research.

Species and numbers of animals expected to be used

- Mice: 4500 over five years

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We wish to use mice for these experiments as evidence suggests that the genetically altered models (mouse) that have been generated and normal animals are best suited to model the aspects of neurodegenerative disease that we wish to investigate. There are no other suitable vertebrate models that are available to us, which will be suitable for our proposed investigations. Although we have also considered using zebrafish but this option has been dropped because of low efficiency of our virus carriers in this model. We plan to use embryonic (for neuronal cultures), neonate and adult rodents.

Typically, what will be done to an animal used in your project?

1. Therapy administration (e.g. cisterna magna)

Gene therapy is way of treating diseases caused by changes (mutations) in a person's genetic code (DNA) by giving them a corrected version of the affected gene. As a gene therapy lab focussing on neuroscience the majority of our animals are modelling diseases of the brain and nervous system. In order to deliver our gene therapy treatments to the brain of our animal models we use a technique that allows us to inject directly into the brain. This is called an "intra cisterna magna" injection. The cisterna magna is a small space between different parts of the brain, located at the back of the skull. Intra cisterna magna is usually performed in neonate pups through the skin (i.e. without surgery). This procedure is performed on a single occasion per animal when using virus carriers.

In some cases we may also need to use more than one way of delivering our treatment. In this case we may need to inject directly into the brain (intra cisterna magna) but also into the blood stream as well (intravenous). We would then follow these animals over time using the methods described beow to record how the disease is changing or whether our treatment is working. These recording could be performed weekly or monthly during the course of the study. Again the end point of all these studies would be sacrificing the animals to collect organs that would be studied in the lab.

2. Functional observations:

Neurodegenerative diseases often lead to symptoms that effect the ability to control our muscles and movement. This is one symptom that we can model in our mouse models, which we call "motor function". However, we need ways to look at and record these changes. One way we do this is by using a piece of equipment called the "Rotarod". This is basically a small cylindrical rod attached to a motor which can spin at different speeds. We can train the mice stand on this rod as the speed of its spin increases. Normal healthy mice that are not modelling any particular disease are able to remain balanced on the rod even as the speed and time increases. However, in mice that are modelling neuronal injury and have lost neurons and muscle function they are often unable to remain on the rod, eventually falling off. This small drop does not harm the mice, but it allows us to measure the time they lasted or the speed at which they fell off. We can then see if our treatments are working. The rotarod test is performed monthly over a period up to 15 months.

Another way we study our mice as their symptoms change is by scoring them. This “score” can be based on a number of things, including their movement, how quickly they are able to turn themselves over when placed on their back, or also something called “clasping”. Clasping is when a mouse pulls in its back legs close to its body when held by the tail. This is something that normal healthy mice do not do. Again this does not harm the mice, but it is something that has been seen in lots of different mouse models of “neurodegeneration” or loss of brain cells.

Loss of brain cells can also lead to other symptoms such as changes in behaviour and activity. We can look at this using the so called “open field test”. This is essentially a specialised camera which can track and follow an animals movement when that animal is placed into a new environment. Differences in how mice explore or move around this new cage can compared between healthy mice and our disease models. Another assessment of behaviour and activity is the “marble burying” test. This works by placing glass marbles into the sawdust of the mouse’s cage. We can then measure how many marbles are buried or left alone. Differences in how many are buried might suggest changes in activity, or how anxious or compulsive a certain mice might be.

While we can study our mice visually by these methods, unfortunately the final point of many of our studies is to look at changes actually within the brain of our mice: has this mouse lost brains cells in a certain area, has our treatment stopped brain cell lost, do cells look healthier after treatment? These are all questions that can only be answered by collecting the brain tissue and studying it in the lab. For this reason the final part of our studies involves sacrificing the mice to collect the brain as well as other organs.

What are the expected impacts and/or adverse effects for the animals during your project?

The animals liable to develop neuronal injury will be allowed to develop some clinical signs of disease, eg tremor, abnormal walking, clasping. All proposed mouse models develop mild/moderate phenotypes. Certain background strains can carry non-MND related phenotypes. For example, 20% FVB mice (including non-transgenic and transgenic mice) develop seizures which can be fatal. Mice where a seizure is observed will be individually identified and will be humanely killed. Mice with seizures will not be included in our procedures/studies. All animals will be killed after completion of each 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)?

The proposed models are limited to no more than moderate severity. The proposed mouse models develop mild severity (50%) (e.g. SPG15 and SPG47) and moderate phenotype (50%) (e.g. Syngap1 and C9orf72). Mice are monitored closely to optimize their housing conditions to make them as comfortable as possible.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We have carefully considered the extent to which these experiments could be replaced by studies involving use of cells in dishes. We test all our vectors in human cells. Only promising virus carriers are then tested in animals. It is important to consider the complexity of the brain and spinal cord and the connections and interactions that cells that innervate muscle make with other cell types in the spinal cord and at the neuromuscular junction (connection between nerve terminal and muscle). It is impossible to replicate this in vitro, even using cells collected from embryos (e.g. mouse embryos).

Which non-animal alternatives did you consider for use in this project?

We are currently developing non-animal alternatives for our future projects. The applicant is leading on the following major projects:

- We secured funding to develop 3D printing for spinal cord cell models. If successful, these models would help minimise the use of animals by screening therapies.

- The applicant is currently leading a consortium of 34 partners (academic institutions and pharma companies). One of the aims of this consortium of 34 partners is to develop better models for testing advanced therapies. we are using plates to generate 3D cell models for therapy testing.

Why were they not suitable?

We have also considered using zebrafish for our in vivo modelling. However, this was not possible because of the low efficiency of our vector carriers in zebrafish.

The 3D cells models are still being developed and will be fully characterised. If successful, we will then initiate their use to screen potential therapies.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise

numbers consistent with scientific objectives, if any. These 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 proposed numbers of animals were estimated based on inputs from biostatisticians and guided by the numbers used under current license.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

i. Our general approach is to test our therapies in in vitro systems (cells in dishes) prior to more formal testing in mice. We plan first to test the efficiency of our vector carriers in cells in dishes and having observed a positive effect, only then move on to transgenic approaches.

ii. Our studies will usually be staged with the aim of obtaining key results on the efficacy of our approach, and in order to perform statistics to determine an appropriate sample size for subsequent investigations. Our protocol 1 is allowing us to undertake pilot studies with small number of animals to identify promising approaches before progressing to large studies.

iii. We aim to design experiments that maximise use of animals for data collection. Thus we aim to use the same animals for behavioural/neurological testing, and biochemical and pathological studies where possible.

iv. We will continuously monitor our experimental results and refine the design of experiments and the number of animals that might be required to provide statistical relevance. Where necessary we will consult biostatisticians for confirmation that our approaches use the minimum number of animals necessary.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

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, details of the experimental material, and the size of the experiment (number of groups, numbers of animals/group); and an outline of the method of analysis of the results (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 with the treatment differences that are to be estimated).

We aim to design experiments that maximise use of animals for data collection. Thus we aim to use the same animals for behavioural/neurological testing, and biochemical and pathological studies where possible.

Protocol 1 of our application will enable us to carry out pilot studies with small number of animals. Only promising therapies will be progressed towards large proof-of-concept studies.

We use mice that behave in a very predictable way, allowing us to use fewer.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 wish to use mice for these experiments as evidence suggests that the genetically altered models (mouse) that have been generated and wild type animals are best suited to model the aspects of neurodegenerative disease that we wish to investigate. There are no other suitable vertebrate models that are available to us, which will be suitable for our proposed investigations.

Our protocols cause the least pain, suffering, distress or lasting harm consistent with achieving our scientific objectives and are limited to no more than moderate severity.

We decided not to use models with severe phenotypes.

Why can’t you use animals that are less sentient?

Although we have also considered using zebrafish but this option has been dropped because of low efficiency of our virus carriers in this model.

We already made a decision not to use models with severe phenotypes.

We also use neonate mice (P1-2) for our initial studies. This age allow injection of our therapies through the skin (without surgery). Undertaking these injections in adult only would require surgical procedures.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Our protocols cause the least pain, suffering, distress or lasting harm consistent with achieving our scientific objectives and are limited to no more than moderate severity. We decided to refine procedures from severe to moderate.

We work actively to minimize suffering. We have developed close links with the animal care staff at our facility and we actively involve them in decision-making.

Close monitoring will be in place for animals under our studies. We will observe the animals for body weight, morbidity, mortality, injury and intact of food and water supported by close monitoring of body weight. Any animals observed to be in poor health will be identified for further monitoring and possible anticipated study termination (based on scores listed under endpoints sections). Where any animals show signs of poor health or distress the NACWO and/or NVS will be informed and consulted.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The surgical procedures 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 (2017) and PREPARE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our strategy is summarised as follow:

- Attend event, conferences and webinars where advances in the 3Rs are discussed and shared.
- Share our knowledge gained from Funding Committee/Boards: e.g. the applicant has been acting as a Panel member of relevant funding bodies. This is an opportunity to keep awareness of new technologies and advances in this area.
- Have been successful in attracting funds to establish 3D model systems. The current programmes led by the applicant will likely lead to advances in this space.
- We will continue our efforts to collaborate with other investigators to secure further funds to address the 3Rs.
- Seek amendment to the PPL to implement these advances.

60. Causes of and treatments for spinal muscular atrophy

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

Spinal muscular atrophy, Therapy, Energy, Whole-body

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?

To understand the causes of abnormal energy storage and production in various organs in spinal muscular atrophy (SMA), a neuromuscular childhood condition, and to evaluate the benefits of new and relevant treatments, including, but not limited to, drugs currently being used for other conditions, specific diets and/or the manipulation of genes. Where complementary action is anticipated, therapies may be combined.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Spinal muscular atrophy (SMA) is a genetic disorder that is caused by loss of function of a gene called survival motor neuron (SMN), leading to severe muscle loss. While three new drugs that increase the amount of SMN have recently been approved to treat SMA

patients, they unfortunately do not fix all symptoms that impact the quality of life of individuals living with SMA. Some of these symptoms are associated with how different organs in the body (muscle, heart, liver, fat, pancreas) control how we store and use energy for day-to-day activities, termed metabolism. Indeed, we have recently shown that changing the diet of SMA children that had received an SMN drug improved several symptoms associated with abnormal metabolism. Others have also recently reported that liver problems were still present in SMA children and adults that had received an SMN drug. While we know that metabolism is dysfunctional in SMA, it is difficult to define the role of each individual organ to the overall defects as all metabolically active organs in the body communicate with each other constantly to control and maintain whole-body health. In addition, it is still not clear how metabolic dysfunction is associated with the severity of the disease. We therefore propose to better understand the abnormalities in different SMA tissues and in metabolism in general. We will aim to identify commercially available drugs and diets that are predicted to fix these abnormalities.

Our ultimate goal is to help and support the SMA community (patients, families, researchers, clinicians, industry) by providing essential and new knowledge that will increase our understanding of the disease, improve the design of treatments, identify relevant treatments to reduce metabolic symptoms in SMA patients and help other muscle-wasting conditions characterised by muscle loss and metabolic defects.

What outputs do you think you will see at the end of this project?

New information on how abnormalities in energy storage and use in various organs occur in SMA.

Identification of new therapeutic targets to improve SMA symptoms.

Efficacy assessment of therapeutic interventions that act on newly identified therapeutic targets (e.g. repurposed drugs, diets, genetic manipulation).

Information that can guide the future development of treatments to better manage the abnormalities in energy storage and use that occur in SMA.

Several publications.

Who or what will benefit from these outputs, and how?

SMA patient and family communities as well as the wider muscle-wasting patient and family communities: by providing them with new and relevant information on what causes symptoms observed in SMA patients and on affordable and accessible options to treat and improve these symptoms as well as as by providing them with new knowledge that could be applicable to energy abnormalities that occur in other muscle-wasting conditions characterised by loss of muscle(mid-/long-term).

SMA researchers, clinicians and healthcare providers: by providing them with new information on causes of symptoms and relevant treatments that they can further research in their laboratories and clinics and/or use to improve clinical care assessment and management (mid-/long-term).

Industrial partners: by providing them with new molecular targets and evidence-based demonstrations of beneficial therapeutic interventions to help them design, develop and commercialise new treatments for SMA (long-term).

The wider scientific communities: by providing them with new knowledge that could be applicable to their field of research (short-/mid/long-term).

How will you look to maximise the outputs of this work?

The results generated in this study will be disseminated via open access peer review publications and by presenting our work at numerous international and national conferences. We will also engage with fundamental researchers, clinicians and industry partners by sharing our progress and findings via our University, Google Scholar and ResearchGate profile pages, our professional Twitter account as well as various outreach/public engagement events. Through our expansive collaborative network, research dissemination endeavours and public engagement activities, our work will thus reach a great number of fundamental researchers and clinicians. As our research progresses and is communicated to an increasing number of individuals, new UK and international collaborations will be fostered, thus further expanding the breadth of academic beneficiaries impacted by our work. With these strategies, we will ensure that our research objectives and results reach numerous academic beneficiaries and importantly, give them an opportunity to engage with our research activities aimed at better understanding and improving health in SMA patients.

Species and numbers of animals expected to be used

Mice: A maximum of 3600 mice is estimated to be used during this 5-year project, which includes breeders and experimental mice (genetically modified and wild type).

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Transgenic SMA mice are used at different ages and disease manifestations to help us better understand disease pathophysiology and develop new treatment strategies. The use of transgenic SMA mouse models that recapitulate the human condition genetically and behaviourally is invaluable to the advancement of our basic knowledge of disease causes and progression and assessment of therapeutic interventions. In particular, to determine how different organs as well as energy storage and use are influenced by the disease state, a whole biological system is required as these symptoms interact and depend on each other. The life stages (neonatal, juvenile, adult) correspond to the different disease stages (pre-symptomatic, early symptomatic and late symptomatic). These depend on the specific mouse model used and treatment received.

Typically, what will be done to an animal used in your project?

Typically, an animal will receive a daily treatment 5 days after birth (typically by gavage once a day), weighed daily from birth, right itself from its back daily and be killed by Schedule 1 once it manifests a defined humane endpoint.

The Smn2B/- SMA mice are indistinguishable from their healthy littermates up until 10 days after birth, at which point they start exhibiting a slower growth rate and weight gain,

an increased righting time as well as shorter tails and smaller ears.

Eventually, untreated Smn2B/- mice will slowly start losing weight and reach their humane endpoint around 20 days after birth.

The MyoD-iCre SmnF7 SMA mice have a delayed disease onset and are indistinguishable from their healthy littermates up until about 18 weeks, at which point they start exhibiting decreased performance in motor function tests and decreased weight. Eventually, untreated MyoD-iCre SmnF7 SMA mice will slowly start losing weight and reach their humane endpoint around 200 days after birth.

What are the expected impacts and/or adverse effects for the animals during your project?

The adverse effects expected to most commonly occur during this project are weight loss and and reduced motor function due to the SMA phenotype, mild pain and/or stress due to injections or gavage, stress due to daily handling as well as maternal rejection following handling of neonatal mice. These adverse effects are minimised by very close daily monitoring of mice with designated score sheets, handling mice with the recommended NC3Rs cupping and tunnel techniques as well as selecting appropriate humane endpoints.

Expected severity categories and the 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 uses GA and WT mice for breeding and maintenance of transgenic and control lines.

Of these, we expect:
- 100% subthreshold severity

Protocol 2 uses GA and WT mice bred in protocol 1, Of these, we expect:
50% mild severity corresponding to some experimental WT animals (e.g. one single procedure), some experimental healthy littermates (e.g. one single procedure) and experimental pre-symptomatic SMA mice.

50% moderate severity corresponding to some experimental wild type animals (e.g. daily procedures over several days/weeks), some experimental healthy littermates (e.g. daily procedures over several days/weeks) and experimental symptomatic SMA mice.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The use of transgenic animal models that recapitulate the human condition genetically and behaviourally is invaluable to the advancement of our basic knowledge of disease causes and progression and assessment of therapeutic interventions. In particular, to determine how different organs as well as energy storage and use are influenced by the disease state, a whole biological system is required as these symptoms interact and depend on each other. For this project, we will use different mouse models (Smn2B/-, MyoD-iCre SmnF7), when relevant, that represent different severities of the conditions (moderate and mild), similar to what occurs in the human condition, and that reflect >80% of the SMA population.

Which non-animal alternatives did you consider for use in this project?

When relevant, non-animal cellular models (e.g. C2C12 cells, LHCN-M2 cells, 3T3-L1 cells, NSC-34 cells, H9C2 cells, FL83B cells) will be used to assess efficacy, activity and toxicity of the treatments before they are evaluated in animals.

Computer programmes will be used to identify commercially available treatments that are predicted to improve the multiple organ and energy abnormalities in SMA.

Why were they not suitable?

The non-animal cellular models and computer programmes will allow us to select the therapeutic interventions in a scientifically sound manner. However, to understand how SMA leads to multiple organ and energy defects over the life course and to evaluate the impact of therapeutic interventions on these symptoms during disease progression, a whole-body system such as a representative mouse model 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?

Using the NC3Rs Experimental Design Assistant, PREPARE Guidelines, G*Power software, previous literature as well as the number of animals used in our most recent PPL with a similar level of research activities.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Using the NC3Rs Experimental Design Assistant, PREPARE Guidelines and previous literature.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Efficient breeding strategies to produce the maximal number of experimental mice of the

desired genotype (genetically modified and wild type).

Collecting blood and multiple organs from one single animal for multiple analyses.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use genetically altered mice that mimic the human SMA muscle-wasting condition and healthy control mice. We will use two genetically altered mouse models that are well-established, have previously been characterized and are widely used in the field. One genetically altered mouse model displays SMA symptoms due to genetic defects affecting all cells and tissues, while a second model displays SMA symptoms affecting only muscle tissue. The muscle only genetically altered mouse model has a less severe disease presentation, with delayed onset and slower progression, compared to the whole-body mouse model. Having 2 models allows us to prioritise, when scientifically relevant and feasible, the genetically altered animals with milder symptoms. Whilst muscle-wasting is recognised as the predominant disease trait, SMA is a whole-body disease in humans. Therefore, for some investigations and therapies not limited to the muscle pathology, the use of the whole system model will be necessary

The behavioural tests are commonly used and they have been designed to provide scientifically valid data whilst minimising adverse effects such as exposure to pain, suffering, distress or lasting harm to the animals due to mouse handling and being exposed to new and unfamiliar environments. For example, the recommended NC3Rs cupping and tunnel handling techniques are used to move animals from cage to behavioural test as well as the lengths of the behavioural tests have been optimised to provide scientifically valid data whilst minimising the time the animal undertakes the behavioural test.

Why can’t you use animals that are less sentient?

Less sentient species (e.g. c.elegans, zebrafish) have been considered but are not appropriate for our research programme as they do not accurately reflect the neuromuscular and/or metabolic organisation and functions in humans and/or the defects that have been reported in SMA patients.

More immature life stages (e.g. embryonic) are not appropriate for this research programme as different post-natal stages (neonatal, juvenile, adult) are required to assess the progression of the whole-body symptoms during the life course of the animal.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Use score sheets to monitor the well-being of animals.

Place animals in a warming chamber to keep them warm before and/or after treatment.

Keep treated and improved SMA animals longer with dam to provide sufficient warmth and feeding.

Provide wet food at the bottom of the cage for treated and improved SMA animals for easy access to nutrition.

Remove male from cage when dam is pregnant to minimise stress in the cage when the litter is born.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will adhere to the resources provided on the NC3Rs website and the PREPARE Guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

By accessing the information on the NC3Rs website: https://nc3rs.org.uk/experimental-design
By participating in relevant workshops.

By updating the NC3Rs Experimental Design Assistant diagrams as the project progresses.

61. Development of Non-Invasive Delivery Technologies

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Drug delivery, Age-related Macular degeneration, Inflammatory disease

Animal types	Life stages
Rats	adult
Rabbits	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to demonstrate the effectiveness of penetration enhancers made of membrane penetrating polyamines (MPPA) and their ability to deliver therapeutics into the eye and through the skin without the need for injections. This is crucial for the treatment of a range of chronic disease such as age-related macular degeneration, rheumatoid arthritis and Crohn’s disease amongst others. These diseases currently require multiple injections on a daily, weekly or monthly basis, often for the entire duration of the patients life. Utilising the MPPA technology will eliminate this requirement.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 ocular diseases such as Age-related Macular Degeneration (AMD) take up a significant portion of the NHS budget. Age-related macular degeneration is a prevalent cause of vision loss in Europe and the US. Current treatments for AMD involve monthly intravitreal injections into the patient's eye for a minimum of 5 years, this is extremely

distressing for patients and has side effects such as retinal detachment that requires ocular surgery. These side effects are a direct result of the injection method rather than the therapeutic itself. The MPPA technology would allow the administration of these drugs as eye drops instead of injections. This will alleviate many of the issues associated with this treatment approach, increasing patient compliance as well as saving costs for the NHS.

Chronic inflammatory diseases such as rheumatoid arthritis and Crohn's disease necessitate frequent injections of TNF-α inhibitors such as adalimumab. Patients with these conditions have to self- administer injections on a weekly or sometimes even daily basis. This has a significant impact on patient wellbeing and leads to poor patient compliance. Using the MPPA technology to offer topical applications of the therapeutics currently used in clinic has the potential to significantly increase patient compliance, increasing patient wellbeing as well as resulting in a reduction in NHS costs associated with treating patients with inadequately controlled conditions.

What outputs do you think you will see at the end of this project?

The main result of this project will be to showcase the effectiveness of the membrane-penetrating polyamine (MPPA) system as a new drug delivery method for both ocular and dermal applications. The project aims to publish the findings as academic outputs, which will not only contribute to the development of the project but also provide a knowledge base for other researchers in the field.

Additionally, specific product outputs will help support the development of the intellectual property already filed and enable its translation into a commercially viable product for our organization. We are currently in discussions with interested pharmaceutical companies to progress towards clinical trials using the data obtained from in vivo experiments as soon as possible.

Who or what will benefit from these outputs, and how?

In the short term there will be two key benefits from this project: it will provide impactful publications and valuable experience for the participating academics, thereby boosting the profile of early career researchers. It also has the potential to support an application to the MHRA for a clinical trial of the delivery vehicle.

In the longer term, the impact of this project on patients with Chronic conditions could be immense. In the case of Age-Related Macular Degeneration this technology has the potential to empower patients by enabling them to self-administer their treatment at home instead of traveling to the hospital every month. This is particularly important for patients who are still employed and can't afford to take time off work every month for treatment.

For conditions such as rheumatoid arthritis and Crohn's disease a key cost for both conditions is poor patient wellbeing and financial burden to the NHS. These can both be alleviated by the removal of the requirement of invasive injection procedures. Poor patient compliance results in uncontrolled disease progression. This has significant reduction in patient wellbeing, often results in attending emergency care and can have high treatment costs to rectify. If this technology proves successful it will eliminate these social and financial burdens to both patients and healthcare providers.

Furthermore, this technology may be critical as the aging population continues to increase, potentially overwhelming the NHS's ability to provide treatment for all patients. There may not be enough specialist trained ophthalmologists available to provide injections to every

patient in need, making this technology an even more valuable asset for managing chronic conditions.

How will you look to maximise the outputs of this work?

We aim to maximize the impact of this project by collaborating with academics and companies working in this field to optimize the use of the data we collect. We aim to rapidly disseminate the results of our tests, whether positive or negative, to inform the academic community and support other researchers who are developing technologies in this area. By working collaboratively and sharing our findings widely, we can ensure that the outputs of this project have the greatest possible benefit for advancing research in this field.

Species and numbers of animals expected to be used

- Rats: 1100
- Rabbits: 550

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We have selected rats as literature resources have demonstrated that this animal model is suitable for drug delivery experiments. We also have the expertise both by the PPL holder and the PIL holding staff in the group to support this work. We have chosen rats as they have a larger surface area on the cornea than mice which will maximise the delivery of the therapeutic into the eye. While age-related macular degeneration is an ageing disease we are carrying out proof of concept studies so we do not need to use aged rats. However, we will need to use adult rodent rather than immature animals to ensure that the eye is fully developed. Adult eyes will also have a greater tissue mass which will give us more material to analyse than rats in earlier life stages. For the dermal protocols, again we need to use adult animals as we need to ensure the dermal layers are fully developed. As the conditions we are examining are not linked to a specific age group, again, we do not need to use aged animals.

We have also chosen rabbits because the larger eye size will be key to determining efficacy of delivery to the posterior segment in ocular indications. The path length in a rabbit eye is closer to a human eye than a rat. This will give key information about the translation of the technology from animals into humans. Studies will not be carried out in rabbits until efficacy is established in rat models first.

Typically, what will be done to an animal used in your project?

For both ocular and skin protocols the animals will have a treatment applied non-invasively.

In the ocular protocol: Animals will be restrained and have a single eye drop applied to each eye. The animals will then be rehoused until the required time point is reached. The animals will then be killed using a schedule 1 killing method and the tissues harvested.

Tissues will be analysed posthumously using ELISA and Immunohistochemistry.

In the dermal protocol: Animals will be restrained and have a small area of their skin shaved. Following this, the formulation will be applied topically to the skin. The animals will then be rehoused until their scheduled timepoint is reached and will then be killed using a schedule 1 killing method and the tissues will be harvested. Tissues will be analysed posthumously using ELISA and immunohistochemistry.

What are the expected impacts and/or adverse effects for the animals during your project?

We have carried out preliminary studies on our previous licence which has shown that the formulations did not cause any inflammation, irritation or distress to the animals eyes or to the skin around the eyes. It is concievable that some formulations may cause irritation, this will be experienced by less than 1% of 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)?

It is expected that animals will experience mild severity. It is possible that some therapeutics could cause irritation or inflammation and these animals would experience moderate severity. Less than 1% of 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?

We have carried out a number of screening studies both in in vitro and ex vivo models. However, for drug delivery, blood pressure and blood flow are key to determining the efficacy of the drug delivery system and there is no way to test this effectively without using animals. This efficacy is essential in order to fully quantify the system prior to entering into clinical trials.

Which non-animal alternatives did you consider for use in this project?

We have carried out a number of studies funded by the animal free research centre which have allowed us to build model screening systems from human cells. Firstly, we use a complete cell monolayer and apply the MPPA and therapeutic to determine the ability of the MPPA to deliver drugs across a cell membrane. The cells are specifically identified based on the delivery type e.g. ocular, dermal. Once this has been established we move onto testing the MPPA in biological membrane. We isolate a membrane from a chicken egg and use it a specific testing apparatus to determine that the MPPA can deliver the therapeutic across the membrane.

Once this is established we move into tissue specific ex vivo testing.

For ocular delivery we will then use ex vivo porcine eyes which are obtained from local slaughterhouses. The therapeutic is applied to the corneal surface and then each tissue in the eye is analysed. For dermal delivery we use full thickness pig skin, again from local slaughterhouses. All of this screening is completed prior to animal testing in vivo.

Why were they not suitable?

The nature of the eye is that it is a very dynamic system with fluid pressure for aqueous humour and blood flow which has a significant effect on the ability for a delivery system to penetrate into tissues. The most obvious example of this is blink rate and tear film. However, blood pressure and blood flow for systemic clearance are also important factors which are not considered in an ex vivo model.

While ex vivo dermal protocols are useful in showing that the therapeutics can penetrate through the skin. We cannot use them to show that we have achieved systemic delivery of drugs. Also, the ex vivo skin does not have blood flow or blood pressure. These can both change the permeation of drugs and therefore the ex vivo model can only be an effective screen to prepare for in vivo studies rather than replacing 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?

We previously held a licence which we have carried out proof of concept studies on.

Due to the pandemic we weren't able to complete all of the studies. However, the data obtained has allowed us to carry out statistical modelling to determine the animal numbers. We have also published ocular delivery studies which has been peer reviewed on the number of animals used for to determine successful ocular delivery.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

To ensure that our technology can progress to clinical trials while minimizing the number of animals used in each experiment, we consulted with statisticians and sought input from the MHRA.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

In this approach we are aiming to maximise the utilisation of tissue in each experiment by designing protocols that allow us to use both eyes of each animal while minimizing any potential impact from delivery to one eye on the other.
For both the ocular and dermal models we will employ sharing of tissue between

experiments and with other groups to reduce the number of animals required for control groups.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will be using 2 animal types and 2 protocols.

In the ocular model we will only be using topical administration to the eye this will ensure that the animals do not experience suffering as part of an invasive procedure.

The applications will be specifically formulated for the tear film by optimising parameters such as pH and ion concentration to reduce the possibility of irritation.

In the skin model, again we will only be applying treatments topically to the skin we will not be using any invasive procedures this is designed to reduce any animal suffering. As described above, the excipients in the formulations will be carefully selected to reduce any adverse effects.

In both models we have carried out extensive pre-screening of excipients and therapeutics in vitro to refine the samples as much as possible.

Why can’t you use animals that are less sentient?

We will carry out the initial studies in rats as these are classed as less sentient than rabbits. Only once our proof of concept studies which have demonstrated delivery to the desired ocular tissue or through the skin is established in the rat model will we move into rabbits to ensure the same effect is observed in the longer path length of both eye and skin in the larger animal.

For these studies we need to use adults of the species as we need to ensure that their eyes are fully developed. However, in order to refine the project we will use young adult animals. As we are examining delivery of drugs rather than efficacy in disease model young adults can be used.

Insects or fish are not suitable as they do not have the appropriate ocular structure equivalent to the human eye.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will ensure that all necessary monitoring will be carried out. In our proof of concept studies the only things observed during monitoring was transient, brief distress from

restraint. Animals will be held by fully trained staff and restraint will be for less than 3 minutes. For ocular indications, the eye drops have been formulated to be compatible with the tear film which significantly reduced the chances of this includes parameters such as optimising pH an ion concentration. If any adverse signs are observed then monitoring will be increased and pain management can be applied. If inflammation or irritation are observed for more than 24 hours then the animal will be given a humane endpoint using schedule 1 methods.

For dermal studies the animals will be restrained for less than 5 minutes to allow for shaving the area and the application of the formulation. Animals will be held by trained staff and restraint will be for less than 5 minutes. If the animal experiences any nicks or cuts during the shaving process the animal will not be used for skin absorption studies and will or will be given a humane end point and tissue taken post humously for control tissue. If there is any inflammation or irritation from the shaving or the application. If inflammation or irritation continues for greater than 24 hours then the animal will be killed using a schedule 1 method.

We will regularly review the scientific literature to explore emerging technologies and models that could potentially replace the use of live animals in our research.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All experiments will be carried out according to the ARVO best practice guidelines for the use of animals in vision research(https://www.arvo.org/About/policies/arvo-statement-for-the-use-of-animals- in-ophthalmic-and-vision-research/).
Prior to all experiments we will consult the PREPARE guidelines checklist to ensure that valuable data will be generated in the experiment. The resulting data will be published in Open Access Journals wherever possible and in accordance with the ARRIVE vs 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 about advances in 3Rs by staying abreast of literature in the field to allow us to update protocols where appropriate. We will also attend and present our work at conferences to obtain as much information to feed into this protocol as much as possible.

62. Effect of plastic pollution on the brain

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
- Protection of the natural environment in the interests of the health or welfare of man or animals

Key words

Pollution, Nanoplastics, Neurodegeneration, Blood-brain barrier, Environment

Animal types	Life stages
Mice	neonate, juvenile, adult, pregnant, embryo, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to discover if plastic particles (e.g., nano/microplastics) can cross from the blood into the brain, accumulate, and cause the brain to stop working properly (e.g., memory problems, brain cells dying). It will also be investigated if plastic particles can get into the brain more easily during certain illnesses (e.g., type 2 diabetes, neurodegenerative diseases such as Alzheimer's disease).

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

There is a global crisis of plastic pollution in the environment. Plastic can fragment into

nano/microparticles (i.e., nano/microplastics) and be taken up by humans, animals and other living creatures. It is imperative that there are investigations to understand the effect of nano/microplastics as there is evidence nano/microplastics cause damage to cells in the body (e.g., inflammation, cell death). A key area of concern is the brain as inflammation and cell death are associated with brain toxicity and neurodegeneration (e.g., Alzheimer's disease).

What outputs do you think you will see at the end of this project?

Generating New Information:
This project will generate new information about how pollutants (e.g., microplastics) may be aggrevating or even causing metabolic and neurodegenerative diseases. This knowledge can be added to what other scientists know about these diseases to work out why and how these diseases are triggered. Importantly, this information can be used to inform government environmental policies.

Publications:
The impact of plastic pollutants in our environment and the effects on the human body upon ingestion of them is rich and untapped research area for publication.

Therefore, this is opportunity to publish findings in high-quality, open-access journals.

Products:
Finding out new information about the impacts of global plastic pollution may help identify unknown factors that may be exacerbating metabolic and neurodegenerative diseases and lead to the development of new approaches to preventing these diseases. Discussions can be held with companies to help translate knowledge or support the development of new products and approaches to removing plastics from our environment (e.g., technology to enable plastic clean-up from water- ways, development of new 'plastics' from plant sources).

Who or what will benefit from these outputs, and how?

Outputs from this project will benefit the wider scientific community researching metabolic and neurodegenerative diseases by adding to the growing knowledge about why and how these diseases happen. Outputs from this project can also be used as evidence of the links between environmental pollution, microplastics and metabolic and neurodegenerative diseases. This can help inform the public and policy makers about how pollution and lifestyle choices can lead to neurodegeneration and is relevant for public health advice on how to maintain good brain health.

How will you look to maximise the outputs of this work?

Publication of 'negative' results:
In accordance with guidelines on pre-clinical research, negative findings will be published in specialised journals (e.g., F1000, JCBFM Negative Results).

Disseminating new knowledge to networks:
New findings will be disseminated to relevant networks including Alzheimer's Research UK, Alzheimer's Society, Diabetes UK and NIHR Dementia Researcher. This work can also allow for the development of new networks, for example, working with members of the Dementia Research Institute, which will lead to enhanced collaboration and sharing of new knowledge, experimental methods, and equipment.

Public Outreach:
Knowledge from this project will be used to raise awareness of environmental pollution and metabolic and neurodegenerative diseases, to improve understanding of the impact of not looking after our climates, and increase public pressure on government to develop policies to protect out environments and fund research into metabolic and neurodegenerative diseases.

Species and numbers of animals expected to be used

- Mice: 1250

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mouse models are an appropriate model to study as they have a blood-brain barrier which is comparable to humans. Furthermore, mouse models allow for the study of the ingestion of nano/microplastics and their movement through the blood vessels to the brain and other organs. Mouse models of metabolic and neurodegenerative diseases as they allow the study of diseases which usually take years to develop in humans within months. Furthermore, these studies will explore specific genetic or environmental changes within a controlled environment without confounding factors. Whilst it is valuable to have the data from humans, mouse models are vital to be able to understand diseases as they are developing, carry out functional studies, explore disease-driving mechanisms and for evaluating new potential medicines.

Typically, what will be done to an animal used in your project?

The animals used in this project will be studied over time (usually for 3 months but potentially up to the maximum of a year) to understand if and how nano/microplastics are entering the brain and if this is more likely to occur in diseases which affected the blood-brain barrier. Animals will ingest nano/microplastics through either eating, drinking, or having them injected so they enter the blood stream. This may happen every day for a maximum of 4 weeks. Animals with genetic changes may also be used to understand if having certain diseases which already affect the blood-brain barrier can allow nano/microplastics to enter the brain more easily or if plastics can aggravate diseases. The effect of nano/microplastics on the brain can be assessed by measuring the cognitive ability of the animals and more in depth analyses can be carried out in brain tissue at the end of the study to measure how much plastic is in the brain and if there are any other effects such as inflammation. Interventions may also be applied (e.g. medicines) to assess if any negative effects of plastics can be slowed-down or stopped.

What are the expected impacts and/or adverse effects for the animals during your project?

Most of the mice that are bred act and look normal and will not show any adverse effects. Giving mice the nano/microplastics shouldn’t have side effects, but they may need to be given by gently passing a soft tube into the stomach or by injection. Mice given

nano/microplastics are not expected to show any adverse effects. Some mice will have small changes in their genetic make-up that cause them to become obese and have high levels of blood sugars. This will make them drink and urinate more and can make them less active. Other mice will have genetic changes that mean that, as they age, they show signs like those seen in neurodegenerative diseases. However, as we study what happens in early disease, these signs will be mild. We will take small blood samples to check glucose levels. At the end of experiments in live mice, the mice will be killed with tissue and blood samples collected for post- mortem analyses to investigate how much plastic may have built up in the brain and other organs.

Overall, most of our animals won't experience more than a "mild" severity associated with brief needle prick(s) associated with injection or blood sampling or due to slight changes in behaviour due to deterioration of some of the nerves in the brain as part of the neurodegenerative disease models.

Some animals will reach a "moderate" severity because they will show signs of frank diabetes, such as having very high blood sugar levels, being very thirsty, and urinating a lot.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

1250 mice (over 5 year period):
Breeding and maintenance of animals: 750 (mild: 500, moderate:250)
Animals to be used in studies: 500 (mild: 400, 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?

Animal models are required to recapitulate the ingestion of nano/microplastics through multiple organs (e.g., stomach, intestine, liver, kidney, vascular system and brain). Replicating a model which is required to investigate how nano/microplastics are ingested, taken up by organs and potentially cross the blood-brain barrier, is not possible in vitro. This is because we are modelling how nano/microplastics are present in food or water and get in to the body by being eaten or drunk. These nano/microplastics would then end up in the gut. Using animal models allows us to track if the nano/microplastics can enter the blood stream from the gut, get into organs which filter the blood such as the kidney and liver, and let us investigate if the nano/microplastics can work their way through these organs and ultimately end up in the brain. It is not currently possible to replicate this complex multi-organ journey in vitro.

Additionally, in vitro models that accurately replicate the multi-cell interactions of the blood-brain barrier (e.g., cross-talk between different cell types including endothelial cells, astrocytes, pericytes, vascular smooth muscle cells) do not currently exist. The in vitro BBB models which are currently available have many limitations and do not model the complex structure and dynamics which make the blood-brain barrier so unique, such as altered expressions of relevant cell biological transporters, ligands and enzymes that give the blood-brain barrier its properties.

Finally, in humans, metabolic and neurodegenerative diseases are often established and develop over months and years, meaning replicating these timelines in vitro is not possible. Furthermore, uptake of nano/microplastics is to be studied in disease models of metabolic and neurodegenerative diseases. Replicating the complex and progressive conditions that occur in these diseases is extremely difficult however animal models provide a whole system that allow us to examine multi-organ diseases which can be studied overtime and at different stages of the diseases.

Which non-animal alternatives did you consider for use in this project?

A number of approaches and models were evaluated for carrying out the required experiments to answer our scientific questions. In vitro culture models of the blood-brain barrier have some advantages over in vivo models, including cost effectiveness, easy to source, wide-range of applications, and are applicable to high throughput screening.

Aside from rodent models, there are a number of other in vivo models that can be used to investigate the blood-brain barrier, including zebrafish, fruit flies and C. elegans. These models have advantages over rodent models as they are less sentient, most cost effective, reproduce quickly, can be genetically manipulated more easily, and are simpler to house.

Why were they not suitable?

In vitro models (e.g., cell lines) have been considered. However, in vitro blood-brain barrier models are cultured in artificial environments that often lack physiological conditions unique and essential to the blood-brain barrier, including exposure to blood flow, cell polarity and crosstalk from multiple cell populations (e.g., endothelial cells, astrocytes, pericytes, vascular smooth muscle cells). For example, the current blood-brain barrier organ-on-a-chip applications, which are some of the most developed with flow and tubular cell organisation to replicate a vessel, currently can host approximately 3 cells types, however, whilst the blood-brain barrier primarily consists of endothelial cells, pericytes, and astrocytes, these cells also form connections with vascular smooth muscle cells, neurons, and microglia and also interact with red blood cells and numerous immune cells found in the blood. These cell connections and interactions could not be include in the organ-on-a-chip. Furthermore, there a several different types of endothelial cells within the blood-brain barrier (i.e., artery, arteriole, capillary, venule and vein) which have a very specific and unique structural order along the blood vessel which cannot be replicated in vitro. These different endothelial cell subtypes have different properties which are key to functions, for example, the will take up nutrients and remove waste at different rates, functions which we are starting to understand are affected in metabolic and neurodegenerative diseases. Furthermore, these chips have only been developed to include bi-directional flow, a false environment, as the blood-brain barrier in vivo is uni-directional which is integral to developing cell polarity and key to the development of blood-brain barrier structural properties, such as tight junctions.

Lastly, you cannot carry out cognitive tests on in vitro models.

Other in vivo models were considered, including zebrafish, fruit flies and C. elegans. However, these models have rudimentary versions of the blood-brain barrier, which lack human translatability as they do not recapitulate the human system as closely as a mammalian model. For example, these models have different levels of permeability and types of transporters expressed. It is also more difficult, or not possible at all, to carry out assays which would inform about disease progression, including physiological and behavioural assays.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 experiments, studies are designed taking into account information from others working in our area of science and from our previous results so that we can work out, as far as possible, the minimum number of animals needed to get a clear answer to the question we are asking. We use statistical tools, such as power calculations, to help us work this out. Pilot data will be used to determine variability of groups and set group sizes. Studies will be randomised and blinded where possible. If mice cannot be randomised to a condition group (e.g., because they have a set genetic modification) then mice will be randomised to other factors where appropriate (e.g., intervention vs no intervention). If it is not possible to blind a study during data collection (e.g., when working with obese animals where a physical characteristic reveals which group a mouse belongs to), the experiment will be blinded during data analyses.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Experimental design will use PREPARE and ARRIVE guidelines and checklists. Furthermore, experimental design tools such as the NC3R's Experimental Design Assistant (EDA) can be used to assist in experimental design to minimise the number of animals required to answer scientific questions, refine methods to reduce subjective bias, and apply appropriate statistical analyses. Where appropriate, mice will be used to collect multiple in vivo data sets (e.g., mice can undergo behavioural tasks and metabolic measurements) and will have repeated measures recorded over time (e.g., at 3 months and 6 months old). Mice will also have tissue collected from them for ex vivo studies.

Statisticians advice will also be followed for analyses and power calculations, as well as software tools including IBM SPSS Statistics, GraphPad Prism and GPower.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Breeding of mice will be kept to a minimum, consistent with sufficient numbers for studies and supply of tissue. Breeding colonies will be set up as appropriate taking into consideration (i) expected Mendelian ratios for transgenic mice and (ii) actual outcomes observed from breeding. Breeding strategy will be continually refining keep numbers at a

minimum consistent with the scientific objectives and the statistical power necessary for examination of each hypothesis.

Sample size calculations will be performed for each set of experiments, taking into consideration published data from similar studies and our own published and preliminary pilot work. Sample sizes will be calculated using standard deviations with parameters set for a significance level of 0.05 and a power level minimum 0.8.
For longitudinal experiments (e.g. long-term exposure to micro-plastics or mouse phenotype characterisation), we take into consideration losses due to fighting, however, good husbandry techniques should minimise these effects.

We will endeavour to use the minimum number of animals to achieve significance for each end point and to allow, wherever possible, multiple experimental outcomes from a single animal (e.g. by well defined and standardised protocols and the banking/sharing of tissues).

For each experiment, a detailed protocol and study plan is first produced and agreed with named training and competency officer and named veterinary surgeon and a diary of events maintained. In all cases, tissues will be collected and archived for subsequent analysis by both our group and others working in similar areas of research requiring tissue from animal 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 will induce metabolic and/or neurodegenerative diseases in many of the animals in our studies. Where possible we will do this non-invasively, for example, by using a high-dat diet to make animals obese or due to genetic changes (e.g., mutations to give mice type 2 diabetes or Alzheimer's disease). These methods minimise any potential stress or pain.

Cognitive and memory tasks may be carried out multiple times. Many of these tasks will involve the exploration of a new or familiar environment or object. This experience can be enriching for the animal and beneficial to the animal's well-being.

Multiple procedures may be performed in any single animal at multiple time point. This reduces the number of animals required but can result in several procedures being carried out in any one animal. A balance needs to be sought between reduction in animal numbers and number of procedures performed. Where any animal showing distress during a protocol, it will be removed from the study and, if appropriate, euthanized, and discussed with the named veterinary surgeon to identify whether modifications are required to minimise reoccurrence of the issues identified.

Why can’t you use animals that are less sentient?

Aside from mouse models, there are a number of other *in vivo* models that can be used to investigate the experimental aim, including zebrafish, fruit flies and C. elegans. However, these models have rudimentary versions of the brain cellular networks, which do not recapitulate the human system as closely as a mammalian model. It is also more difficult, or not possible at all, to carry out assays which would inform about brain health, including physiological and behavioural assays.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Experimental methods have been and will continue to be refined where possible. For example, we always use non-aversive handling methods for our mice and let them become familiar in advance with the person who will be performing any procedures. This training allows for reduced stress during procedures such as behavioural tasks or tail blood samples where following handling, mice do not need to be restrained.

If an animal might be hurt or distressed by a procedure, we will place them under an anaesthetic, so they are asleep for it. If we can, we try and do no procedures while the animal is alive, we just use tissue in the lab (but unfortunately this is only suitable for some types of study), or we do the experimental interventions when they are asleep under a non-recoverable anaesthetic to reduce any awareness, discomfort, or pain. If there is the chance of pain then analgesics can be used pre- emptively, during a procedure, and/or after.

All testing will be performed by experienced members of staff, following standardised protocols thereby minimising welfare costs for the animal whist also reducing variability and ensuring that the data produced is of a high quality and reproducible. New staff are rigorously trained. All studies are carried out following recommended guidelines under the guidance of the local veterinary team and in a facility with highly trained staff.

We keep animals in compatible groups as far as possible and we take measures to make sure that the mice can express their natural behaviours as far as possible by providing enrichment in the home-cage environments such as “houses”, tubes, chew blocks, different types of bedding to make nests and hanging suspended fixtures (like tubes and “swings”) from the cage lid for climbing.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

I will continue to apply best practice guidance for experiments acquired from LASA, NC3R, and other appropriate websites, including the PREPARE guidelines, ARRIVE guidelines and the Experimental Design Assistant.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will regularly visit the NC3Rs website and others recommended by the local named information officer. I will also keep up to date with Research Animal Training eModules.

63. Neural mechanisms of memory persistence

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Learning, memory, pavlovian, instrumental, neurobiology

Animal types	Life stages
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to study how recalling a past experience can promote or inhibit the memory of that event. Our work will focus on the brain mechanisms supporting or inhibiting memories of either unpleasant or rewarding events.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

My work is relevant to anxiety disorders (e.g. post-traumatic stress disorder or specific phobias) and drug addiction treatment since these are characterised by the persistence of aberrant associative memories. I use laboratory animals that allow us to investigate memory persistence or inhibition mechanisms, with the goal of influencing the development of novel therapies to address memory- related psychiatric disorders in humans. Building on my previous work, my research aims to identify druggable cellular and sub-cellular mechanisms supporting the persistence of positive or negative memories. My previous findings on protected animal species are now beginning to influence human memory research in healthy volunteers and patients.

What outputs do you think you will see at the end of this project?

The main output of this project is further our understanding of the molecular and cellular mechanisms responsible for inhibition or maintenance of memories after retrieval. We will reveal the molecules that are involved in each process and find out if memories of good or bad events are processed differently by the brain.

We will publish our findings on peer reviewed scientific journals, and disseminate them at scientific meetings in the UK and abroad, as well as in public events.

Who or what will benefit from these outputs, and how?

Our work will have different beneficiaries.

The most direct effect will be on our future work and of other scientists working on memory persistence. Our findings will inspire more detailed investigations on memory persistence of both rewarding and aversive experiences.

In the intermediate term we expect our findings to serve pharmaceutical companies and healthcare professionals to develop more effective treatments for anxiety disorders and drug addiction patients, in the UK and worldwide. Such disorders are linked to aberrantly persistent memories. Hence, understanding better how memories can be inhibited will open new avenues for treatments.

Also, as this project is investigating the basic mechanisms of the brain during memory formation and maintenance, our findings will benefit humankind in general, revealing how the brain works when it works well by maintaining valuable memories and inhibiting outdated ones.

How will you look to maximise the outputs of this work?

To reach the full potential of our work, we will establish collaborations with colleagues from the UK and abroad. We will also take the lead in translating the outputs of the project into translational research in humans, both with healthy volunteers (to corroborate our findings when possible) and in patients.

We are comitted to disseminate and publish all the outputs of the project, both positive and negative results. In this way, future projects can build upon our experience and results, without needing to run the same experiments again.

Species and numbers of animals expected to be used

- Rats: 3000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use rats as our experimental species. Rodents are the simplest vertebrate laboratory animals in which sophisticated behavioural assessments can be performed

while retaining relevant neuroanatomical and physiological homology with humans. Since the project main objective is to understand memory mechanisms, and memory is expressed behaviourally, intact awake behaving animals are necessary. Given the difference in memory persistence mechanisms reported for juvenile versus adult rodents, and to keep our findings as relevant as possible to psychiatric conditions affecting human adults, we will only use adult individuals. The use of these animals is supported by the robust and readily observable behavioural output of memory formation, retrieval and extinction, a brain size that allows individual analysis of cellular and molecular events in relation with behaviour, and our extensive experience with these animals. Finally, using an animal close to humans will allow future translation of our findings into human research and development of novel treatments for psychiatric conditions linked to aberrant memory processing.

Typically, what will be done to an animal used in your project?

Typically, animals will learn associations between environmental cues or their own actions and food or threats.

Animals trained with food rewards will have time restricted access to food. They will receive their daily food portion after the behavioural procedures, so they show a higher motivation for learning food-cues associations. Food restriction will only last while the behavioural manipulations are taking place.

Animals trained in threat conditioning will be exposed to a mild footshock co-terminating with discrete environmental cues, once or twice within one single session. These footshocks produce no tissue damage and have no effects on the animal's biology or behaviour, other than their memory expressed upon presentation of the threatening environmental cue.

In some animals we will inject substances systemically (i.e., intraperitoneally) or intracerebrally. For the latter group, animals will be operated to receive viral injections (to label memory neurons) or to implant injector guide cannula for pharmacological interventions that may affect their memory processing.

The experiments will typically last between 2 to 4 weeks. The number of procedures will range from one (behavioural training) to three (behavioural training, surgery, in vivo drug administration).

What are the expected impacts and/or adverse effects for the animals during your project?

Our behavioural manipulations will have no general effects on the animals' health or behaviour, but they are designed to change their behavioural response to specific environmental conditions. This is how we can make sure animals remember a past experience.

In operated animals, we do not expect any behavioural, physical or physiological alteration, other than in the context of the specific target memory we will be investigating (see above).

The surgical interventions will produce moderate pain during recovery, and this will be treated accordingly.

Importantly, since this project is focused on behavioural manifestations, our procedures

are highly refined to minimise discomfort to animals. Rodents that do not feel well cannot learn or demonstrate they remember specific past experiences.

Expected severity categories and the 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 all our procedures is moderate.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Unfortunately, revealing the neural mechanisms underlying memory formation, persistence and inhibition could only be done by performing experiments *in vivo* using behaving animals. Rodents are ideal laboratory animals. They are small and easy to handle, and more importantly, present robust behavioural manifestations, which helps minimising sample sizes required to obtain meaningful data. Also, their brains present anatomical and neural organisation similar to the human brain, which contributes to identify neural mechanisms of brain functioning with translational potential.

Which non-animal alternatives did you consider for use in this project?

I have considered using ex-vivo, in-vitro or in-silico approaches as alternatives to research with animals. Memory could also be studied in humans.

Why were they not suitable?

Memory is a complex phenomenon arising from events taking place at different organisational level, from molecules to behaviour. The alternative approaches of ex-vivo, in-vitro or in-silico are not suitable since they lack (and could not model) the fundamental properties and interactions present in behaving animals with functioning nervous and other systems.

Working with humans would give us access to the behavioural and some neural aspects of memory, but could not address our research questions regarding the neural mechanisms of 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?

I have calculated the number of animals that are used on average by postdocs and PhD students using the experimental tools proposed here. One postdoc could use around 200 animals per year. A PhD may use around 100 animals per year. I envisage my research group will be composed of two postdocs and two PhD students in the near future, depending on funding. So, overall, I estimate my research group will be using around 600 rats per year, that is 3,000 in the five year period of this license.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

To use the minimum number of animals required to obtain statistically valid data and meaningful biological relevance we will design our experiments very carefully. We will always maximise the behavioural and molecular data obtained from each animal, minimising distress. We routinely calculate effect sizes from pilot studies or previous experiments (published or our own) to determine the minimum number of subjects required for reliable and reproducible effects. We will use, whenever possible, within-subjects analysis to maximise statistical power keeping animal numbers to the minimum.

The behavioural tasks proposed here have been used in the field for several decades, and have been refined to minimise distress while ensuring reproducibility and robust behavioural measurements.

Animals will be allocated randomly to each experimental group by using online random number generators. In some cases, we might allocate animals ‘pseudorandomly’ to ensure that all experimental groups present a similar pre-treatment value of the dependent variable. We aim to fully automate data collection, but when this is not possible the observer will be blind to any treatment information. In all cases the statistical analysis is decided along with the design of each experiment, and before data collection and the actual analysis.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We ensure the minimum number of animals are used by using good experimental design, seeking appropriate statistical advice and following the ARRIVE 2.0 guidelines (DOI: 10.1371/journal.pbio.3000410).

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 for this project. The behavioural paradigms will include threat and appetitive conditioning. The methods will comprise pharmacological or genetic interventions via intraperitoneal, or intracranial administration of substances. When surgeries are necessary we will implement the best practice in the field to minimise pain and suffering, ensuring group sizes are kept to a minimum to assure robust and reproducible results.

For threat conditioning we will use the minimum footshock intensity to motive robust behavioural readouts, without producing lasting harm or suffering, other than the formation of a long lasting threat memory towards specific environmental cues.

Why can’t you use animals that are less sentient?

Rodents are biologically and behaviourally similar to humans that the outcomes of the project will be relevant to the understanding of human memory processes, in health and disease. Using other organisms that also have memory but are evolutionary more distant to humans compromise translation and impact on human health and disease.

Also, there is a wealth of literature in rodent's memory mechanisms that we will use to help reducing the number of experiments to achieve meaningful results with translational potential.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

After every experiment we will critically evaluate the procedures to seek out any ways to improve our models to reduce harm to animals.

In case of operated animals we will monitor on a daily basis to detect any adverse effects. We will implement post-operative care with analgesic drugs for a set amount of days post-operation, as well as provide with a calm and warm recovery area in the early hours after surgery. Animals will recover alone for a minimum of 12 hours, with provision of water and wet mashed food. To maintain animals welfare, all our operated animals will then be moved on to pair housing.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

I will follow the guidance provided in the following documents/sources:
Guidance on the Operation of the Animals (Scientific Procedures) Act 1986 (Home Office).

Code of Practice for the Housing and Care of Animals Bred, Supplied or Used for Scientific Purposes (Home Office).

Advisory notes on recording and reporting the actual severity of regulated procedures (Home Office).

Use, Keeping Alive and Re-use (Home Office).

The Harm–Benefit Analysis Process (Home Office).

Guiding principles for and undertaking Aseptic Surgery (2nd Edition April 2017, LASA).

The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research (PLoS Biol 18(7): e3000410. 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?

Reading new papers using these animal models will help me keep updated with the latest refinements on these techniques. Also, I am subscribed to the NC3R newsletter and receive and read it on a regular basis to stay up to date with the latest developments.

64. Protein misfolding diseases: pathogenesis and intervention

Project duration

5 years 0 months

Project purpose

- Basic research
- 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 misfolding diseases, Cell death

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 better understand human diseases resulting due to protein misfolding and aggregation, through mimicking those disease in cultured cells & mice. We aim to further utilise this understanding to change the course of diseases by pharmacological and/or genetic interventions.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

With the aging of the general population, the prevalence of neurodegenerative disorders is expected to rise dramatically in the next few decades, with a resultant increase in the societal and financial burden of these disorders. In recent years, we have made significant progress towards this aim and discovered strategies to help cells survive with undesirable

proteins. We have demonstrated the relevance of our findings in few animal models of human diseases. This establishes the proof-of- principle that the strategies we identified in cells have big potential towards prevention of different protein misfolding diseases.

So far, we showed that pharmacological treatment with two compounds prevents development of symptoms in three mouse models of unrelated protein misfolding diseases. Importantly, our new preliminary results show that the efficacy of our compounds is not limited to these three conditions (unpublished work). This licence will enable us to continue assessing the benefit of modulating stress response pathways in a variety of mouse models of diseases that arise from accumulation of misfolded proteins (neurodegenerative diseases) or more broadly when protein quality control fails (such as diabetes). The impact of this approach is potentially huge.

What outputs do you think you will see at the end of this project?

The work proposed here will give further insight into the benefits of our generic approaches against protein misfolding. We anticipate that we will ameliorate a broad range of models of human diseases associated with misfolded proteins or protein quality control failure. Identification of strategies, that can rescue from the protein quality control failure, is of a great importance. The new information will be disseminated through publications in peer-reviewed journals. These approaches have big potential to progress into clinical trials.

From our proof-of-concept results obtain in the course of our research we know that the approaches that we develop are of a significant relevance. They are of a big interest in the scientific community, they are also an attractive target for drug development. As an example, our molecule Sephin1 is now tested in Phase 1 human clinical trials.

We recently discovered another strategy to modulate protein quality control pathway which showed promising results. This led to discovery of new compounds to ameliorate protein misfolding diseases. Therefore, the outcome of our ongoing research can lead to more drug candidates which may enter to human clinical trials, in future.

Who or what will benefit from these outputs, and how?

The benefit from the work described here is to provide further evidence that modifying the natural cellular defence systems against misfolded proteins can slow down human diseases in order to identify pathways that can be exploited therapeutically.

How will you look to maximise the outputs of this work?

We have a strong expertise in the protein quality control system. We are well experienced in the whole broad range of techniques required to develop our approaches. We have been successfully using all methods required in the course of our previous studies. We have a strong support from our highly experienced animal facility team. Our proof-of-concept published results and preliminary unpublished data show the validity and feasibility of our approaches.

Species and numbers of animals expected to be used

- Mice: ~25000 mice may be required.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Human diseases resulting due to dysfunctional protein quality control have been well recapitulated in mice. This enhanced the understanding of process of disease development in great detail. Mice, as research models, are reasonably easy to handle, breed, train and manipulate. Moreover, the commercial availability of various transgenic mice strains for different human diseases made it the top choice for our research.

We plan to use adult and/or aged mice depending upon research questions. For example, in case of neurodegenerative diseases in transgenic mice, due to rapid progression of disease, younger mice are used but if studied in wild type (or transgenic strains with slow progression of disease), aged mice will be used. We will maintain breeding colonies and generate new transgenic lines while cryopreserving existing transgenic lines which means that all life stages will be used in this project.

Typically, what will be done to an animal used in your project?

Mice will be housed in a social environment whenever possible and provided with environmental enrichment such as chew sticks, fun tubes, nesting material and platforms. Mice may be subjected to altered food composition, food deprivation and weight monitoring to study diseases like diabetes.

Some mice may be aged beyond 15 months and up to 2.5 years to allow monitoring of disease progression. These mice may show age associated phenotypes like obesity, loss of vigour, discolouring, thinning and loss of hair, change in social hierarchy pattern, worn teeth, atopic dermatitis etc. Other than above said signs of ageing, mice may also show signs of neurological symptoms like, progressive hind limb paralysis, change in abilities like memory and/or learning, motor coordination defect etc.

Some mice may undergo behavioural tests to check for neurodegeneration or cognitive impairment such as motor function or memory tests. These tests will be of non-invasive (e.g. Open field, Rotarod, Object recognition etc.) type based on scientific question being addressed. In any case, health status of mice will be carefully monitored before testing and only healthy individuals may be subjected to these tests.

We may administer substances by a variety of routes, choosing the most suitable and least invasive route possible. Some of the routes will be non-invasive such as food or water. Other routes such as injection (under the skin, into the peritoneal cavity, into a vein, into a muscle) or oral gavage will cause mice transient pain and/or transient stress. Following the administration of substances, some mice may be aged to observe the possible development of neurodegeneration in brain and spinal cord, in which case these mice will experience weight loss and pain due to progressive hind limb impairment.

Other than ear biopsy collection for genotyping purpose, live mice may be subjected to blood collection which can cause transient pain and/or transient stress.

At the end of experiments, mice will be humanely killed, and samples collected for

analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

Mice will be maintained in social environment and environmental enrichments will be provided to improve their wellbeing. Mice, being monitored for disease progression, will be kept until they develop disease symptoms like weight loss or hindlimb paralysis but will be culled latest at this stage. Some mice strains may require ageing up to 2.5 years (or more if scientifically justifiable) to allow neurological phenotype to develop. These mice may show expected levels of old age-related adverse effects. Aged mice showing adverse effects beyond expected levels will be culled.

Some mice will be administered with pharmacological substances which might cause intermittent distress, but no lasting adverse effects are expected. In most cases substances will be administered by oral route.

In all cases behavioural tests that will be applied to mice are non-invasive and are not expected to cause any lasting distress or harm. These tests are essential to determine the efficacy of treatment in the disease prevention and is required to validate a tested compound as a potential future therapeutic. At the end of the procedure mice will be humanely killed to collect tissues. Post-mortem tissue analysis will be conducted to monitor diseases pathology as well as drug efficacy.

The genetic modification of some mice might lead to sudden unexpected deaths in a limited number (5%) of mice that are not preceded by any prior disease symptoms, therefore not possible to predict. In the past years, we developed refined protocols and monitoring procedures that enabled us to decrease the incidence of mice found dead suddenly and unexpectedly. We continue to search for further refinements. Nevertheless, we are unable to predict and prevent unexpected deaths completely.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Approximately 60% of mice will not experience any pain and are classed as sub-threshold severity.

Approximately 10% of the animals will experience some pain which will be mild severity such as an injection.

Approximately 30% of animals will experience pain such as progressive back leg disability and are classed as moderate severity.

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?

We have discovered novel, powerful and straightforward approaches to rescue cells from failure of protein quality control. These results have a potentially huge impact for human health because a large number of diseases arise when protein quality control fails. Prior to being used in humans, there is a legal requirement for virtually all potential disease modifiers to be tested in animal models for the disease(s) in question. The mouse is best suited for this work since, of all existing animal models, mouse models, due to the brain structures similarity as well as the extent of the disease recapitulation, are highly relevant to the human diseases.

Which non-animal alternatives did you consider for use in this project?

We have used cultured human cells as non-animal alternative as they are the best starting points to test disease modifiers due to direct connection with real disease. We have done as much as possible and will continue in the future to carry out pilot experiments in cell lines or in ex vivo cultures but whilst they will provide some useful information, cultured cells cannot fully replace mouse models.

Why were they not suitable?

Cell lines and ex vivo cultures do not provide physiological conditions nor the complex interactions amongst different cell types. Ex vivo cultures represent some interactions between cell types within the tissue, but they don’t recapitulate the drug metabolism presented in vivo. The work in mice we propose to carry out is essential to validate our discoveries and may have a big impact on human health.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number estimation was done considering our experience of colony size, maintenance breeding, longitudinal studies, and surplus generation using our transgenic mice. We maintain ~15 transgenic strains of mice and based on planned experiments which requires ~5000 mice per year.

Some transgenic lines (e.g. HD82, SOD1) are maintained at relatively large numbers because only males are fertile with a very limited breeding window and short lifespan. These males are paired with females of other strains (e.g. WT females) for mating. To maintain such lines, more breeding is set to generate enough transgenic breeder males required to maintain the colony. The breeding goes further up when experimental cohorts

need to be generated.

Some mice will be used for breeding to maintain the colony. Some mice will be bred for tissue collection for ex vivo work. Some mice (in groups of 10 to 15) will be aged to allow disease pathology to develop prior to tissue analysis of a time course which will usually have four to eight time points.

Some mice will be given pharmacological modifiers (groups of 10 to 15) and tissues collected for analysis.

Some mice will be used for behavioural testing (in groups of 10 to 15) with or without substance administration to study the disease progression. In few cases (e.g. nTr-20 strain), to detect subtle behavioural differences, groups of 15-20 mice may be used.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will use minimum number of animals necessary to get statistically significant results. Number of mice needed will be evaluated based on previous studies, pilot experiments or power analysis.

Statistician will be consulted.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Mouse breeding will be carefully monitored to ensure that minimum or no surplus animals are generated. To reduce variability and bias, mice will be randomly assigned to experimental groups. Experiments will be conducted in a blind fashion.

Cryopreservation will be used to preserve important lines and to remove the necessity to hold stock for extended periods.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The animal models of human protein misfolding diseases such as neurodegenerative diseases which we use express the human proteins known to be involved in the diseases (huntingtin, tau and alpha- synuclein) and exhibit the essential features of the human diseases and as such they are the best models available for our studies.

We have a monitoring programme in place for all our animals to assess health status in order to avoid animals experiencing unnecessary adverse effects. There is an enhanced

monitoring programme for older animals and animals which may develop weight loss and/or hind limb impairment to minimise suffering.

When new lines are generated and bred for the first time, animal technicians will be specifically informed, and the first litters carefully monitored. Any untoward phenotype will be discussed with the NACWO, NVS and if appropriate, the Home Office inspector.

All animal experimentation conducted under this project licence will comply with the local animal usage guidelines. This document has been adopted by the local ethical review process in order to inform researchers on refinement of procedures and to provide practical recommendations on various aspects of animal experimentation (i.e. routes of administration and appropriate volumes of administered substances, severity classification of specific clinical signs, appropriate anaesthetic regimes etc.)

Why can’t you use animals that are less sentient?

To understand the disease progression mechanism of human protein misfolding diseases, we need a mammalian species which can be easily genetically manipulated, recapitulate process of disease progression and easy to handle. Mice are the best sentient representative species which fulfils all those requirements. Also, another great advantage of mice is that they have a substantial life span of approximately 3 years. Therefore, they can be aged to study slow progression of protein misfolding diseases such as neurodegenerative diseases.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Some of our mice develop motor impairment as they age, and these mice will be closely monitored for difficulty satisfying hunger and thirst and for weight loss. Where appropriate food and drink will be provided on the cage floor.

In view of the progressive decline associated with ageing there will be increased vigilance both in monitoring indices of general health (weight, grooming, reactivity) and additional observations designed to detect specific problems such as the development of tumours.

For administration of a substance the route of administration will depend on the solubility and bioavailability of the compound and the least invasive route that is suitable will be chosen.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We use the NC3Rs website to ensure that we follow best practice. We use The Experimental Design Assistant (eda.nc3rs.org.uk) on the NC3Rs website to check the design of our animal experiments and ensure that we have robust and reproducible data.

We follow the PREPARE (norecopa.no/prepare) planning guidelines and the checklist of the ARRIVE (arriveguidelines.org) guidelines to maximise the quality and reliability of our publications.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We keep up to date with the current literature and subscribe to the NC3Rs newsletter to get the latest updates. We attend seminars and conferences where the 3Rs are discussed. We encourage the sharing of best practice between animal technicians.

All animal welfare issues are addressed, and solutions implemented following discussions between the animal technicians, the NACWOs, the veterinarian and PIL holders.

65. Genetic and environmental effects on behaviour in fish

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Stress, Aggression, Personality traits, zebrafish, Behaviour

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 overall aim of our research is to determine the influence of genes and environment on the behaviour of zebrafish. Specifically, we will determine the impact of potentially stressful stimuli, mainly isolation, on the behaviour of zebrafish, for example, how anxious, aggressive or sociable they are, and the mechanisms causing any changes.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 will give us a greater understanding of the impact of early life stress, specifically isolation, on aspects of fish behaviour and the mechanisms that are involved. The research may provide insights into early life stress that are applicable to humans,

given the similarity in their genes. Furthermore, zebrafish are kept in aquaria throughout the world and it is important to understand the environmental conditions that can affect their behaviour and welfare.

What outputs do you think you will see at the end of this project?

Outputs include gaining new information on the impact of environmental factors early in life on the behaviour of individuals and the mechanisms involved. The findings of the proposed research will be published in high impact peer-reviewed journals.

Who or what will benefit from these outputs, and how?

The research will be of interest to the scientific community, specifically those working in the area of early life stress. In addition, this research will be of interest to those working on aspects of fish welfare and could feed back into improved husbandry techniques throughout the world.

How will you look to maximise the outputs of this work?

Collaborations will be established and maintained with colleagues from the same institution and with those from different institutions. Research will be disseminated through presentations at conferences, both academic and those specifically on zebrafish husbandry (e.g. those arranged by Aquaneering), as well as open access publications.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 10,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The nature of the work, which is on animal behaviour, necessitates the use of live adult zebrafish. Zebrafish produce lots of embryos and we are able to provide most of their needs easily. Zebrafish have had their genome sequenced and they share many genes with humans. These factors make these fish ideal model organisms to use when investigating behaviours, some of which may be relevant to humans (e.g. Eachus et al (2017)).

Typically, what will be done to an animal used in your project?

This project will involve measuring and assessing the behaviour of wild type and mutant zebrafish. Experiments will expose wild type zebrafish to isolation for short periods of time (60 days maximum) and/or chemical (e.g. predator water) or visual (e.g. predator image)

stimuli and assess their behaviour as adults. Some fish will undergo further isolation (14-21 days maximum) after the initial isolation.

Mutant fish will not be isolated but will have their behaviour assessed. Behavioural tests will measure a variety of behaviours such as anxiety and sociability. Some tests will measure aggression using their response to a mirror image or another individual as a gauge of this.

What are the expected impacts and/or adverse effects for the animals during your project?

The behaviour of the zebrafish will be assessed and some differences between groups are anticipated. However, any fish that exhibit obvious signs of stress e.g. subdued and inactive on the bottom of the tank for more than 24 hours will be removed from the experimental procedure and euthanised. Fish will be monitored throughout the experiments for any obvious signs of stress.

Expected severity categories and the 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 mild

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The nature of the work, which is on animal behaviour, necessitates the use of live animals.

Which non-animal alternatives did you consider for use in this project?

NA

Why were they not suitable?

NA

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Estimations are based on previous research conducted by ourselves on a previous PPL and by other scientists working in the same area (e.g. Fontana et al 2020; Pimentel et al 2021). We estimate that we will need 30 fish per treatment group (43 to account for 30% mortality) to take account of any differences in behaviour, including any differences in sex. Typically for an isolation and re-isolation experiment there are 4-8 treatment groups. Mutant fish behaviour will be compared with controls and wild types that have undergone isolation.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

During the design phase we consulted with previous research and gained additional advice from colleagues in our department and the university who have vast experience in statistical analysis. In addition, we will use the NC3Rs EDA. We will maximise the behavioural data that can be collected from the fish, and at the end of each experiment, when fish are euthanised, we will derive information on cortisol and gene expression from their tissues.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Efficient breeding protocols will be used to ensure that only those embryos that are needed for the experiments will be generated. Tissues of fish euthanised at the end of experiments will be made available to others when possible.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Zebrafish will be used during this project. We propose to expose some animals to isolation and/or visual and/or chemical stimuli and assess their behaviour. In addition, we will assess the behaviour of mutants that may share similar traits to isolated fish. We do not anticipate that animals will be unduly stressed by exposure to these stimuli, but they will be monitored closely for signs of distress and suffering.

Why can’t you use animals that are less sentient?

The nature of the research means that fish will be raised in isolation for limited time frames from the embryo stage until up to or less than day 60 and then as adults for short periods (14-21 days) to determine the impact of this on their behaviour. Very immature stages have a limited behavioural repertoire and so we will assess them at day 60 and above. The organisation of the brain of zebrafish is similar to that of mammals and they have a large behavioural repertoire, not seen in less sentient animals. As a consequence, they are important in translational research (e.g. Kalueff et al 2014).

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals will be monitored throughout the experimental protocol and their behaviour will be assessed at the end. The research is linked to improvements in animal welfare i.e. determining whether impacts of environmental conditions can be mitigated.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Guidance on zebrafish welfare on the NC3Rs webpages and the Norecopa PREPARE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Alerts from the NC3Rs about research, funding and workshops/conferences as well as their resources online. In addition, newsletters from our institution and information from the HO.

66. Engineering antibodies for the treatment of cancer

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

cancer, antibody engineering, antibody-drug conjugate, therapeutic

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 goal of this research is to engineer novel antibody-drug conjugates (ADCs) that have increased therapeutic efficacy, improved drug delivery and reduced toxicity.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Cancer is the cause of 24% of all deaths in the UK, with a much larger percentage of people affected by this disease during their lifetime. Despite the development of better diagnostic and screening methods, the incidence of cancer continues to increase, mainly due to an ageing population, and lifestyle factors. Despite major advances in the

development of diagnostics and therapeutics for cancer, progression to metastatic, and frequently incurable disease, is common. This indicates a pressing need to develop more effective therapies that can, either alone or in combination, be used to destroy or keep in check cancerous cells in the body.

One such approach is to combine proteins called antibodies with highly potent drugs that kill cells. These proteins bind to markers on tumour cells with a high degree of specificity, with substantially lower binding to non-tumour cells i.e. normal tissue. Consequently, the linking of potent drugs to antibodies to generate ‘antibody-drug conjugates’ (ADCs) is expected to result in preferential delivery of the drug to tumour cells whilst not affecting the normal tissue. In this regard, many chemotherapy drugs are delivered to both normal and tumour cells, resulting in unwanted side effects such as digestive system problems. Thus, the use of ADCs in the treatment of cancer is expected to have significant advantages over existing chemotherapies. However, despite the promise of ADCs, their use has been met with problems related to an inability to deliver sufficient drug to tumour cells. In principle, increases in doses could overcome this problem, but despite their preferential delivery to tumour cells, higher doses result in undesirable toxicity towards normal tissues. As a result, multiple clinical trials involving ADCs have led to disappointing results and highly undesirable side-effects.

Our proposed study seeks to overcome the current problems associated with ADCs for the treatment of multiple cancer types. Recent knowledge of how the markers that are targeted by ADCs behave in cancerous cells, combined with methods to alter the behaviour of antibodies and how they interact with these markers, indicates ways of generating antibodies with increased efficiency in delivering drugs to these cells. These approaches will be used to produce a new generation of ADCs that are expected to have superior properties over existing ADCs and, in particular, result in a reduction in unwanted side effects. Our proposed studies will combine the use of protein/antibody engineering with experiments to identify ADCs that have increased activity in killing tumour cells. Prior to testing in humans in clinical trials, it is essential to analyse the therapeutic effects of the ADCs in mouse models of cancer. Our specific objectives are:

1. To develop a new class of engineered ADCs that lead to improved drug delivery.

2. To test the therapeutic efficacy of the ADCs in mouse tumour models.

3. To define the mechanisms that lead to improved therapeutic outcomes in mouse models.

We have been targeting breast tumours, prostate and Burkitt B cell lymphoma tumours, and are now ready to extend to other tumour types as discussed in the Scientific Background section. Our approaches are expected to be generally applicable to multiple other tumour types.

What outputs do you think you will see at the end of this project?

At the end of this project, we expect to generate new classes of therapeutics that have improved activity, with reduced adverse side effects, for the treatment of cancer. The use of ADCs as cancer therapeutics has shown considerable promise, but with current ADCs, problems associated with toxicities have been frequently observed. This results in limitations to the doses that can be used, which in turn reduces their efficacy in killing tumor cells. Our studies propose to overcome these issues by engineering antibodies to result in more effective delivery of the drug component of the ADC to tumour cells.

The strategies that we plan to develop will primarily target breast, urothelial, ovarian, prostate and B cell related malignancies. Importantly, these approaches could be extended to many other tumour targets and types. Consequently, our proposed research has broad general relevance to the treatment of many different types of cancer. The detailed mechanistic analyses that we plan to carry out are also expected to lead to new insight into this form of cancer therapy. In addition, we expect to be able to readily translate our studies in mice to the clinic.

Data from these studies is expected to be of interest to other researchers, clinicians and biopharma. Our results will be disseminated by publications and presentations at conferences. We will also participate in public outreach activities.

Who or what will benefit from these outputs, and how?

The ADCs with improved activity that we propose to generate have the potential to provide curative treatments for cancer. The expected short-medium term outcome of our research is the demonstration that the engineered ADCs have improved efficacy in tumour models in mice. This will be combined with studies to define the mechanistic basis of any improved therapeutic effects that are observed. These studies will be followed up by clinical trials, which will either be carried out through clinical trials and/or out licencing to biopharma.

We have extensive experience in out licencing of technologies to biopharma, combined with collaborations with several biopharma companies that have, to date, resulted in late phase II/phase III trials using antibody engineering technologies developed in our laboratory. We therefore anticipate that a long-term outcome will be translation of the engineered ADCs into the clinic.

How will you look to maximise the outputs of this work?

Dissemination of new, relevant knowledge to the community is one of our utmost goals. We have extensive experience collaborating with several biopharma companies using antibody engineering technologies developed in our laboratory. For example, one of the technologies developed in our laboratory has led to the approval of a therapeutic in the US, Japan and Europe for the treatment of myasthenia gravis.

Data from these studies, including unsuccessful approaches, are expected to be of interest to other researchers, clinicians and biopharma. Our results will be disseminated by

publications and presentations at conferences. We will also participate in public outreach activities.

Species and numbers of animals expected to be used

- Mice: 5616

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The overall goal of this research is to generate antibody-drug conjugates (ADCs) that have reduced undesirable toxicities and improved efficacy as therapeutics. We intend to use adult mice for these as the cellular and molecular interactions of mice are broadly similar to those of humans, allowing us to investigate clinically relevant therapeutic strategies and mechanisms in these animals. There are also numerous tumour models that have been established which are strain-specific and have been widely used in this field of research. Besides, individual mice within a given inbred strain are considered genetically 'identical', which reduces variability, therefore enabling valid conclusions to be drawn from experimental data.

Our goal is to replace the use of adult mice wherever possible with in vitro assays. We carry out extensive testing of antibodies and their corresponding ADCs using cell lines and primary human cells in vitro prior to in vivo studies. Nevertheless, to analyse the biodistribution, pharmacokinetic and therapeutic effects of antibodies/ADCs prior to clinical translation, there is unfortunately no alternative to in vivo modelling at the whole-body level in animals.

Typically, what will be done to an animal used in your project?

Prior to in vivo analyses of therapeutic efficacy, we will carry out extensive in vitro analyses of the activities of the ADCs to identify the lead candidates for in vivo testing.

When lead ADCs have been identified based on in vitro analyses, the following procedures will be carried out in mice:

1. Pharmacokinetic, biodistribution, and imaging studies: these studies are to determine how long the ADC (or corresponding antibody) persists in the body of the mouse, and which tissues it goes to, which is critical to determine the potential for off-tumour toxicity when compared to the ADCs currently available. In the **pharmacokinetic experiments,** mice will be intravenously injected with labelled ADC and blood samples taken at various timepoints to determine the ADC levels. The levels of ADC at the whole-body level will also be analysed at various timepoints using a non-invasive whole-body counter into which mice are placed. For some of these studies, tumour cells will be

injected below the skin in immunocompromised mice, or in their mammary fat pads, under anaesthetic or restraint as necessary, so that they grow as tumours. 4-14 days following delivery of labelled ADC, mice will be euthanized. The **biodistribution studies** will involve the use of mice that have tumours which will be measured at regular intervals until they reach a specific size. Once tumours reach the appropriate size, labelled ADCs will be injected and a maximum of three whole-body count readings will be obtained over 3 days. Mice will be also provided iodine in drinking water prior to the injection of the labelled antibody to reduce thyroid uptake of radiolabelled iodine. 1-3 days following delivery of the ADC, blood samples will be taken, followed by non-recovery anaesthesia and harvesting of tissues/organs to see where the ADC is distributed. For **imaging studies**, similar procedures will be followed as in biodistribution studies, except that the mice will be imaged following delivery of the labelled ADC at different timepoints, under anaesthesia and up to 3 times, at time periods within 1-7 days of injection of labelled ADC, using a whole-body imager. The imaging times will be based on the tumour growth rate and the expected pharmacokinetic behaviour of the antibody/ADC. In addition, in some imaging experiments, mice will be injected with a clearing agent 6-36 h after injection of the labelled ADC to reduce the background and improve contrast of the images. 1-7 days following injection of labelled antibody/ADC, mice will be perfused under non-recovery anaesthesia and tumours harvested to allow quantitation of the (fluorescent) label in the tumour.

2. Therapy and mechanism/tumour penetration studies: Human tumour cells will be injected into immune-compromised mice and measured at regular intervals until they are of the appropriate size for treatment as described in biodistribution and imaging studies. In therapy studies, mice will then be injected with ADC or control at 3-21 day intervals for a maximum of 5 doses. Mice will be monitored at regular intervals for tumour growth and up to 4 blood samples (40 µl each sample) will be taken at 1–2 week intervals, under anaesthetic or restraint as necessary. Mice will be euthanised once sufficient data has been collected to meet the experimental goal. The mechanism/tumour penetration studies is similar to the therapy studies, but 1-7 days following antibody/ADC delivery, mice will be subjected to non-recovery anaesthesia, and tumours harvested for ex vivo analyses.

3. Injection of tumour cells to establish safety and human tumour end-points and to assess overall mice health status. In this protocol, a smaller number of immunocompromised mice will be injected with new cell lines/models and tumour growth monitored as described above. This Protocol is meant to be used for pilot experiments to determine suitable endpoints and ensure no unexpected or excessive harms are evident.

In all studies, mice will be monitored on a daily basis to assess their overall health status. They will also be weighed 1-3 times per week. Mice that show signs of ill-health such as piloerection, hunching, weight loss will be euthanised. Similarly, if the tumours grow up to a previously defined size or are hindering the movement of the mouse, mice will be euthanised.

What are the expected impacts and/or adverse effects for the animals during your project?

In the majority of our proposed experiments, mice will be injected with tumour cells under the skin or in the mammary fat pad. Mice will be carefully monitored and, typically, the tumours will not grow to a size where they interfere with the normal behaviour of the mice such as their ability to move. In the unlikely event that we observe effects of the tumour on the behaviour of the mice, the mice will be humanely killed.

For studies investigating the behaviour of the ADCs in mice prior to therapy testing, the short-term nature of the experiments and regulated procedures that will be used are expected to primarily result in effects of mild severity, with a small percentage of mice showing moderate effects. Typically, therapeutic antibodies or ADCs are well tolerated and will be used at doses that are expected to be well below the so-called maximum tolerated dose, which is the highest dose that can be delivered without observing significant, undesirable side effects. For most of the protocols, mice with relatively small tumours will be used as they are not expected to affect the normal behaviour of the mice.

For the proposed therapy and mechanistic/tumour penetration experiments (two of six protocols), the tumours will be allowed to grow for longer than for other types of experiments to allow us to determine therapeutic effects. Tumour-bearing mice in these experiments will be very carefully monitored for any signs of pain or distress, including behaviours such as abnormal gait, locomotion and/or hunching.

Mice showing such effects, which are expected to be very low in number (5% or less of total), will be humanely killed. Consequently, in therapy experiments, we do not expect the effects to be greater than the moderate severity level, with most effects being in the mild category.

For anaesthesia, we will follow current, best practice methods and do not expect the mice to suffer adverse events. For non-invasive methods such as delivery of antibodies/ADCs by intravenous injection, mice will not be anaesthetised since these procedures result in only transient pain and/or distress.

For harvesting of blood samples from mice, we will use volumes that are substantially lower than those likely to cause adverse effects such as anaemia. Mice will be bled using best practice methods by trained personnel. Based on many years of using similar protocols, we do not expect the effects to be greater than the moderate level, with most effects falling in the mild category. Wherever possible, we will use whole body counting of mice as an alternative to bleeding, since this procedure involves placing the mouse in a cylindrical body counter for around one minute, whilst still allowing some movement of the mouse. This procedure results in minimal disturbance to the welfare of the mouse.

All mice will be humanely killed when the experimental goal or, if sooner, humane endpoint has been reached.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The proportion of mice experiencing various severities are detailed below and, based on our current project licence, we expect the proportions in this proposed programme of work to be the following:

Sub-threshold - 0.6%

Mild - 5%

Moderate - 94.4%

Approximately 94.4% of the mice in our protocols require repetitive blood sampling, repetitive injections and/or anaesthesia which might cause short-term or long-lasting pain, suffering or distress in mice.

Some experiments (eg.: Establishing safety and humane tumour end-points / ~5%) will cause a short- term mild pain, suffering and distress. Even though we have identified this protocol as “Moderate”, we do not anticipate this severity to be reached. However, caution with a new, non-screened cell line must be considered.

We estimate that approximately 0.6% of the animals in this licence will not have an experimental procedure. We aim to minimize unforeseen circumstances as much as possible but unfortunately cell contamination prior to injection, or problems with the ADCs production/purification can occur.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 goal is to replace animals with in vitro methods wherever possible. However, the complexity of the distribution of an antibody or ADC in the body, and its persistence in the blood circulation, cannot be modelled with in vitro systems. For example, possible toxicities due to unwanted distribution of an ADC to normal tissues or organs cannot be mimicked using in vitro cellular assays. In addition, investigation of the therapeutic effects of an ADC in reducing tumour growth, with all the complexities involved in tumour development in the body, cannot be modelled by in vitro systems. In this context we are

committed to replacing mice where possible and the initial evaluation of the developed ADCs will be carried out on cell lines in vitro prior to their use in vivo. At this time, there is unfortunately no viable alternative to in vivo modelling using animals for ADC efficacy evaluation.

Which non-animal alternatives did you consider for use in this project?

Our goal is to replace the use of mice wherever possible with in vitro assays. We will therefore carry out extensive analyses of the engineered antigen delivery vehicles using in vitro assays to assess antigen loading pathways. These in vitro assays will include 2D and 3D culture models for various types of cancer, as well as ELISAs to assess ADC binding activity, accumulation/internalization assays to assess the behaviour of the ADC in tumour cells and microscopy analyses.

We are committed to investigate if there are any new suitable non-animal alternatives available online, by researching on NC3Rs (https://www.nc3rs.org.uk/), NORECOPA (https://norecopa.no/alternatives/) and the ‘Fund for the Replacement of Animals in Medical Experiments’, http://www.frame.org.uk). We also actively discuss this with our research group, peers in meetings, seminars, and conferences to ensure that we are aware of any new or updated best practices. Nevertheless, to analyse the pharmacokinetic, tumour localisation and immunological consequences (e.g. antigen presentation) of delivering the engineered antigen-antibody complexes at the whole body level in mice with tumours or autoimmune disease, there is unfortunately no alternative to in vivo modelling in animals.

Why were they not suitable?

Unfortunately, in vitro systems cannot mimic the complexity of the tumour response after antibody/ADC administration. The complexity of the distribution of an antibody or ADC in the body, and its persistence in the blood circulation, cannot be modelled with in vitro systems. For example, possible toxicities due to unwanted distribution of an ADC to normal tissues or organs cannot be mimicked using in vitro cellular assays. In addition, investigation of the therapeutic effects of an ADC in reducing tumour growth, with all the complexities involved in tumour development in the body, cannot be modelled by in vitro systems.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

A major contribution to our experimental design will be consideration as to how the animal numbers can be reduced. In essentially all experiments, we will use inbred mouse strains, including genetically altered (GA) mice, to reduce variability. This will in turn result in lower standard errors/deviations in the data that is obtained, and lower numbers of animals in each experimental group. Prior to carrying out therapy experiments which, of our protocols for this programme, typically involve the largest numbers of mice, we will take the following steps to reduce mouse numbers:

1. Pharmacokinetic and biodistribution studies with 3-5 mice per group will be performed to identify candidates for therapy.

2. Pilot experiments will be carried out to inform the experimental design of larger studies, including dosing regimen, expected humane endpoint (e.g. for mice in control, untreated groups).

3. Number of animals predicted, based on the experience with our current PPL and respective protocols.

Technologies such as whole body counting for pharmacokinetic analyses and whole body imaging also result in lower numbers of mice since they allow longitudinal follow-up of the same mice over time.

These alternatives avoid the need for increases in mouse numbers due to limitations of the number of blood samples that can be taken from the same mouse, or allow tumours to be followed longitudinally to assess tumour localization of the therapeutic. However, for determination of blood levels of a potential therapeutic, we need to collect multiple blood samples. Our experience in carrying out pharmacokinetic experiments is that longitudinal sampling of individual mice leads to more reliable results than cycling blood collections between different groups of mice. This latter approach would necessitate the use of 3-4 fold more mice, which contradicts the 3Rs requirements.

We will also make every effort to decrease experimental bias and minimise experimental variability. An example of this will be if the humane end points are based on an assessment of the condition of the animal, an experienced and blinded animal technician will be asked for their assessment. Objective measurements such as tumour measurement, whole body counts etc. generally do not need blinded observers. Allocation of mice to experimental groups is carried out by a technician in the breeding colony prior to transfer to the experimental housing and is therefore not likely to be biased. Related to this, we use age and sex-matched mice that have been bred in the same housing for experiments to minimise variability. We will follow the PREPARE guidelines (https://norecopa.no/prepare) for all animal experiments.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We are committed to reduce animal numbers whilst ensuring meaningful and reproducible results. To do this, we follow the Principles of the 3Rs, the PREPARE guidelines (https://norecopa.no/prepare) and have utilised the NC3Rs Experimental Design Assistant in experimental planning (https://eda.nc3rs.org.uk/eda/) as well as the 'PS: Power and Sample Size Calculation programme' (https://biostat.app.vumc.org/wiki/Main/PowerSampleSize) when considering our experiments.

Based on earlier experiments that we have carried out, we have used power analyses to determine optimal sample size. In cases where we have insufficient earlier data, or it is not available from publications of others, we will carry out pilot experiments to determine the expected variability.

An additional measure of reduction is that when new tumour cell lines are introduced in the Facility, they are tested for pathogen contamination by growth in mice prior to use in tumour experiments, to ensure safe introduction into the Animal Facility. We use this period to monitor growth and establish human endpoints, thereby reducing the need of additional 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 will use inbred strains of mice (i.e. with the same genetic makeup) for all of our experiments to reduce the variability that would typically be expected with outbred strains (that are not genetically the same). This results in a need for lower mouse numbers. Our aim is to use the minimum number of mice to obtain statistically robust results that are reproducible across experiments. We will use both our prior experience in carrying out the proposed experiments, combined with power analyses, to determine the numbers of mice that we need for each experiment to draw reliable conclusions. Related to this, we will also use age and sex-matched mice to minimize variability.

In addition, we will carry out smaller, pilot experiments with low numbers of mice, to define doses, tumour growth rates etc. prior to expansion to larger experiments if we have not prior experience with the tumour model and/or therapeutic agent. Nevertheless, the use of tumour cell lines that are well validated in our studies or those of others will form the basis of many of our analyses, and this is expected to contribute to a need for lower numbers of mice.

When possible and where multiple inter-related parameters are to be evaluated, we will also employ longitudinal studies to reduce the need to cull multiple mice at different time points to reach experimental goals. The use of serial bleedings, whole-body imaging, harvesting of tissues/organs for further in vitro assays are examples of these optimization steps to reduce the number of animals required while ensuring their welfare.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The overall goal of this project is to investigate the therapeutic efficacy of engineered antibodies and their corresponding ADCs that are designed to more efficiently deliver cytotoxic drug to target (tumour) cells. Mice are the most suitable of the species and are broadly taken to be good models for the preclinical testing of cancer therapeutics such as ADCs. For example, for the ADCs that are clinically approved, preclinical analyses in mice were crucial steps in their development.

We intend to use mice as the cellular and molecular interactions of mice are broadly similar to those of humans, allowing us to investigate clinically relevant therapeutic strategies and mechanisms in these animals. There are also numerous tumour models that have been established which are strain-specific and have been used in this field of research. Besides, individual mice within a given inbred strain are considered genetically ‘identical’, which reduces variability, therefore enabling valid conclusions to be drawn from experimental data with as few animals as possible.

Our goal is to replace the use of adult mice wherever possible with in vitro assays. We carry out extensive testing of antibodies and their corresponding ADCs using cell lines and primary human cells in vitro prior to in vivo studies. Nevertheless, to analyse the effects of antibodies/ADCs prior to clinical translation, there is unfortunately no alternative to in vivo modelling at the whole-body level in animals.

We expect our studies to result in few adverse events, given that antibody-based therapeutics are widely used in the clinic and are typically well tolerated. We ensure humane end-points are established that minimise the harm to the animal without compromising the accuracy of the experimental data.

Nevertheless, the mice will be carefully monitored and if adverse events are observed, steps will be taken to alleviate them, or the affected mice will be euthanized.

Why can’t you use animals that are less sentient?

Mice are the most suitable species for this research, and they have been shown to be suitable preclinical models for antibody-based therapeutics. As mentioned previously, the cellular and molecular interactions of mice are similar to those of humans, allowing us to investigate clinically relevant therapy strategies and their mechanisms in these animals which are less sentient with very different physiology.

We are only planning to use animals terminally anaesthetised when collecting tumours and/or organs for subsequent in vitro studies (e.g.: imaging and mechanism/tumour penetration studies).

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

To ensure high welfare standards, good animal husbandry, including environmental enrichment, is employed. We also expect our studies to result in few adverse events, given that antibody-based therapeutics are widely used in the clinic and are typically well tolerated. Generally, for this project licence application we expect that the severity of the procedures will be mild for a significant number of mice/procedures, but we have set the maximum limit as moderate as many experiments involve tumour harbouring mice. Nevertheless, the mice will be carefully monitored and if adverse events are observed, steps will be taken to alleviate them or the affected mice will be humanely killed.

Monitoring of animals

Animals are maintained by qualified technicians who have familiarity with many different tumour models. Mice will be monitored daily by the animal house technician(s) and the PIL(s) carrying out the experiments. Measures of distress for the mice will be assessed, such as altered feeding, drinking or mobility, or poor condition that can be detected using other methods such as alterations in facial expression (e.g. see guidelines for this in https://www.nc3rs.org.uk/using-facial-expressions-pain- animals). If one or more animals are anticipated to be close to reaching a humane endpoint (the humane endpoints are well defined for the tumour models that we plan to use), they will be monitored more closely. If a technician finds an animal that is approaching a humane endpoint, the animal will either be immediately culled or the corresponding PIL holder will be informed that the animal needs to be culled immediately (via a Schedule 1 procedure). If mice show signs of distress for which a cause cannot be identified, the advice of the NVS will be sought.

Reagents used

Delivery of tumour cells or labelled antibodies/ADCs will be carried out using a combination of volumes, routes and frequencies that will result in no more than transient discomfort and no lasting harm. Antibodies conjugated to cytotoxic payloads will be used as ADCs in these experiments. The use of these cytotoxic payloads is not expected, based on their wide use in preclinical studies and in the clinic, to result in adverse effects. All cancer cell lines used to induce tumours are grown under aseptic conditions and are tested for mycoplasma contamination on a monthly basis. Cell lines are also authenticated by DNA fingerprinting on an annual basis. Prior to delivery into mice, cells are washed and resuspended in sterile PBS. In some cases, cells may be ‘marked’ by transgenesis or shRNA knockdowns, but this is not expected to have deleterious effects on the recipients. All animals will be monitored daily and if any animal is in pain or distress, or if the tumours have reached the size cutoff described below, it will be killed by a Schedule 1 method. We will seek the advice of the NVS if signs of distress cannot be explained.

The administration of lugol (sodium iodide solution) in drinking water to mice is to reduce the accumulation of radiolabelled free iodine that is released from catabolised, radiolabelled antibody/ADC in the thyroid. Lugol delivery does not result in adverse effects.

Administration of antibodies/ADCs

Generally antibodies/ADCs are well tolerated when used at typical doses. Their properties are such that they are soluble in aqueous buffers, such as phosphate buffered saline, which does not have toxic effects when used as vehicle alone. The antibodies/ADCs will be prepared under low endotoxin conditions to reduce the risk of adverse side effects (e.g. innate cell cytokine release) that can also modify experimental results. We expect to be able to reduce these doses further by engineering the ADCs for improved drug delivery. If we unexpectedly observe evidence of distress/adverse events, the measures taken to alleviate these symptoms are documented in the Adverse events sections for all protocols. It is not only important that the distress to the animal is minimised, but also that mice are not culled from experiments to result in loss of statistical reliability. Any unexplained indication of distress will result in our contacting the NVS for advice.

For pharmacokinetic and biodistribution experiments involving 125-I, mice will be fed Lugol (iodinated water) for three days prior to the start of the experiment and throughout the experiment to reduce iodine accumulation in the thyroid. Lugol does not have any adverse effects on the mice. The use of antibodies or ADCs labelled with near-infrared dyes such as IRDye800CW (near-infrared dye) in whole body imaging experiments is not expected to result in adverse events. Typically, relatively low doses of these agents are used, as described in one of our recent studies.

Whole body counting

For whole body counting, the holder used to insert the mice into the counter, such as an Atom Lab 100 dose calibrator, is cleaned between each mouse to minimise cross-contamination, stress due to pheromones etc. Whole body counting takes about one minute per mouse, and based on our extensive experience with this method, we do not expect the mice to show signs of distress. During whole body counting, mice do not need to be kept stationary during the counting procedure and are placed in a ‘loose fitting’ holder in which they are able to move.

Blood sampling

The sampling of blood/serum will follow NC3Rs guidelines (Table 1) and is not applicable to newborn mice. Pain from bleeding from a superficial vessel such as saphenous vein will be controlled by suitable anaesthesia/analgesic. Bleeding after blood sampling will be controlled by applying gentle pressure, and sample volumes will not exceed the limits presented in Table 1. Based on many years of experience with taking multiple, longitudinal blood samples from mice, we do not expect to see adverse effects. Also, the blood volumes used are not expected to result in anaemia.

Table 1: NC3Rs guidelines for blood/serum sampling in adult mice.

% Blood volume	Volume (µl)	Time scale/frequency
Will not exceed 10%	140	On any one occasion
Will not exceed 15%	210	Multiple bleeds within 28 days
Will not exceed 1%	10	Serial sampling at 24 hour intervals

Whenever possible, we will substitute the analysis of blood samples with whole body counting. However, in some experiments, it is critical to determine the blood levels of an antibody/ADC rather than the whole body levels. Our experience over many years has indicated that the information obtained from whole body counting is distinct to that obtained from determining blood counts. Typically, more rapid clearance is seen from the blood than the whole body, due to extravasation into the tissues. Hence, in many cases, both sets of readings are valuable in analysing the dynamic behavior of the antibody/ADC.

For pharmacokinetic analyses, we typically sample small volumes (10 µl/sample; less than 1% blood volume for an adult mouse) of blood containing radioactively labelled antibodies/ADCs in capillaries for around 12 -16 sample collections (3 on day one, usually immediately after injection and 6 and 12 hours thereafter, 1 per day for subsequent 9-13 days i.e. over a 4-14 day period; the total blood volume will be a maximum of 160 µl). Blood samples (up to a total of 16; 10 µl per sample) will be taken using best practice methods (https://www.nc3rs.org.uk/mouse) from a superficial vessel of mice, such as the saphenous vein, or tail vein with appropriate anaesthetic or restraint as necessary. If restraint is used, restraining devices such as restraint tubes will be cleaned between each mouse change out to minimise cross-contamination or stress due to pheromones. This is carried out longitudinally on the mice within a group, rather than bleeding a subset of mice within each group at each time point which would necessitate the use of substantially larger numbers of mice. In addition, although inbred strains are being used, there is mouse to mouse variability and hence the ability to longitudinally monitor individual mice reduces the total number of mice required.

For all experiments that involve blood sampling, animals will be checked for signs of poor health such as piloerection, hunching, weight loss and culled using a Schedule 1 method if poor health is indicated. However, based on our past experience, we do not expect adverse effects.

Non-invasive imaging

The use of antibodies/ADCs labelled with near infrared or other fluorescent/bioluminescent dyes (including luciferase-expressing tumour cells) will be as recommended by the

manufacturers (e.g. Caliper Life Sciences IVIS imager) and is not expected to result in adverse events. High doses, that are above those that we plan to use, can however result in adverse consequences. Sufficient time periods will be given between each imaging session to ensure that the mice fully recover from anaesthetic and are behaving normally with respect to eating and drinking. The whole body imaging of fluorescent or bioluminescent signals does not involve the use of damaging radiation.

Clearing agents for non-invasive imaging

The delivery of antibody-based clearing agents which comprise engineered antibodies or antibody Fc- fusion proteins, into mice is also not expected to cause adverse events, since analogous agents have been extensively used in preclinical models and these antibody-based clearing agents are currently being used in phase 3 clinical trials. Although antibody-based clearing agents reduce antibody levels, this effect is short-lived, and some of the mice used in these experiments already have very low levels of circulating antibodies due to immunodeficiency.

Anaesthesia

We typically do not deliver antibodies/ADCs or other procedures that are categorized as mild under anaesthesia, since the use of anaesthetics can be invasive and result in trauma. When used, anaesthesia or analgesics will be delivered using protocols described in published sources (e.g.

Flecknell [2023] Laboratory and Animal Anaesthesia, Academic Press). Warming chambers will be used during recovery as needed. Animals will not be left unattended by laboratory personnel during recovery on warming chambers, and the warming chamber temperature will be checked regularly, with independent monitoring of the temperature. For procedures such as whole-body imaging that may involve taking several images at different time points (typically up to three times within a 48-hour time window), mice will be allowed to fully recover between each anaesthesia.

Based on our experience and published literature, the risk and harm of general anaesthesia is estimated to be very small (<1%). In-house experience indicates that animals that make a full recovery 2 hours post anaesthesia with no signs of laboured breathing for example or stress will survive. To account for any aversive effects followed by repetitive anaesthesia, we carefully monitor the animals' weight, breathing and/or time to recover and increase mouse numbers if needed. We recognize that repeated anaesthesia can become aversive and side effects may occur, but we aim to minimize such effects by reducing the number of anaesthetics and using a gentle induction by slowly increasing the concentration of anaesthetics. Animals that fail to recover as described are culled using a Schedule 1 method.

Our procedures will be reviewed regularly to ensure that we are using current, best practice methods.

Tumour models

Many of the tumour models that we plan to use are well characterised and humane endpoints have been identified before the mice exhibit observable signs of pain or distress. The tumour xenografts that we will use are expected to develop as unilateral subcutaneous or mammary fat pad masses, without metastatic spread, which allows facile monitoring of tumour size. In addition, to minimise the possibility of restricted movement, mammary fat pad tumours will be implanted in the 2nd or 3rd fat pad. The pharmacokinetic, biodistribution, imaging and mechanistic studies are of relatively short duration and, based on our past experience, we do not expect to observe poor condition of mice, or for their tumours to reach the size limits that define a humane endpoint (see below). Therapy experiments will be carried out until the differences between control and test groups are significant i.e. the minimum amount of time needed to establish that the engineered ADCs have improved efficacy over the appropriate control ADCs. If one or more mice have tumours that reach the size limits below (typically in control groups), the experiment will be terminated and mice culled using a Schedule 1 method, unless the mice with these larger tumours are clear outliers and can be removed without affecting the statistical analyses. In the latter case, only the affected outliers will be culled. Based on our prior experience, we expect that around 95% of the mice will have tumours below these size limits before the end of the therapy experiment is reached (i.e. significant difference in size between control and test therapeutic).

Tumour size will not be the only humane endpoint used in experiments with tumour-bearing mice, and the Workman guidelines (Workman et al. [2010] Br. J. Cancer, 102, 1555-1557) concerning the use of mice in cancer research will be followed for all experiments involving tumour-bearing mice.

Experiments will be terminated, or individual mice culled (if numbers affected are low), if the tumour interferes with normal behaviour, posture or locomotion. To assess the condition of the mice, we will use the methods described by Ullman-Cullere and Foltz (Lab. Animal Sci., 49, 319-3230[1999]), and a body score of 2 will be used as a humane endpoint.

If one or more mice in an experiment are approaching the critical endpoint, the corresponding cages will be marked for close attention by the PIL holder and animal house technicians. Daily inspections will be performed by animal technicians and PIL holder(s) carrying out the experiment(s). If an animal approaches the humane endpoint or is showing signs of distress and/or suffering, it will be immediately culled by the technician or the technician will inform the PIL holder that it needs to be culled immediately. Schedule 1 methods will be used for culling. However, there are cases where relatively large tumours do not adversely affect the behaviour of the animal. In these cases, it is important to avoid culling animals too early due to size limits, resulting in missing of therapeutic responses that results in invalidation of the experiment and wastage of animals.

Taking these considerations into account, and based on our prior experience in therapy experiments, we will use the following guidelines for tumour size to determine humane endpoints in the pharmacokinetic, biodistribution, imaging, and mechanism/tumour penetration studies, as they are relatively short term and do not involve therapy: 1.2 cm diameter (860 mm3; mammary fat pad tumours or subcutaneous tumours; consistent with Workman guidelines for non-therapy experiments; Workman et al., [2010] Br. J. Cancer, 102, 1555-1577). For therapy studies and for establishing safety and humane tumour endpoints, the following size limits will be taken as humane endpoints: 1.4 cm diameter/1,370 mm3 (mammary fat pad tumours) or 1.5 cm diameter/1,690 mm3 (subcutaneous tumours).

Overall, we do not expect to observe ulceration with the subcutaneous or mammary fat pad tumours. If observed, we expect these mice to be outliers and they will be killed using a Schedule 1 method. If this results in a reduction of group size to the extent that statistically robust results can no longer obtained from the experiment, all mice will be killed using a Schedule 1 method to terminate the experiment. We also have onsite veterinary assistance to provide advice if and when necessary.

However, if we show that our antibodies/ADCs have therapeutic efficacy with starting tumour sizes of 50-100 mm3 (therapy and mechanism/tumour penetration studies) then it may be necessary to show efficacy with larger tumour sizes prior to clinical translation. In these cases, we will start treatment of mice when the tumours are of 100-350 mm3, since earlier culling due to the presence of ulceration might deplete number of experimental groups, impact statistical significance and require further experimental repeats and/or require more mice per group. In this case, ulceration is followed very carefully on the relevant models. If observed, these tumours will be assessed using a scoring system developed by Lloyd and Wolfensohn in the Handbook of Laboratory Animal Welfare and Management. This scoring system considers surface area of ulceration, depth, visual appearance and clinical condition of the mouse. Under this protocol, a score of 3-6 implies that careful monitoring is maintained, a score of 7-10 requires consideration for culling and consultation with a NACWO if necessary, 11-16 requires culling by a schedule 1 method. Mice scoring 7-10 will be culled unless the tumour is not impacting the overall wellbeing of the animal and there is clear experimental justification for it to remain.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

In pharmacokinetic, biodistribution, and imaging experiments involving tumour-bearing mice, the experimental endpoint is clearly defined in each protocol. Based on our experience with several cancer xenografts (such as breast, prostate, B cell lymphoma, ovarian and lung cancer cell lines) that will be used in these studies, combined with the delivery of antibody/ADC when the tumours reach a size of 50-100 mm3, we do not expect the size limit of 1.2 cm diameter (860 mm3; mammary fat pad tumours or subcutaneous tumours; consistent with Workman guidelines for non-therapy experiments; Workman et al., [2010] Br. J. Cancer, 102, 1555-1577) to be reached during these time frames.

Nevertheless, for pharmacokinetic, biodistribution, imaging and to establish safety and human tumour-endpoints to assess overall mice health status, if one or more mice appear to be in poor health or if the tumour size in one or more mice reaches 1.2 cm diameter in outlier mice, mice will be culled using a Schedule 1 method. Indicators of poor health will be reduced mobility, abnormal posture, abnormal gait, piloerection, weight loss of up to, but not reaching, 20% of body weight etc. Mice will be killed immediately as soon as the body weight approaches 20% loss, and 24-48h later for tumour-associated symptoms. Assessment of condition will be carried out as described by Ullman-Cullere and Foltz ([1999] Lab. Animal Sci., 49, 319-323) and a body score of 2 will be taken as a humane endpoint. If such outlier mice can be culled without affecting the experimental output, then this will be done; otherwise all mice will be culled and the experiment terminated at this point in time.

For therapy experiments that are of longer duration when compared to other protocols, experimental endpoints will be implemented when the differences between test therapeutics and control groups become significant i.e. the minimum time period for satisfactory results to be obtained. Typically, for the breast and prostate tumour models that we plan to use, this is expected to occur at 50-70 days post- tumour implantation for ADCs that are therapeutically efficacious. However, for tumours that grow relatively rapidly, such as the HCC1954 breast tumour line, experiments are expected to last for only several weeks. It is possible that significant differences between control and test groups may not be reached before the tumour size in one or more mice reaches a size of 1.4 cm diameter/1,370 mm3 (mammary fat pad tumours) or 1.5 cm diameter/1,690 mm3 (subcutaneous tumours). Based on our current experience we expect that tumours will not reach this size in around 5% of the mice before statistical significance is reached. If these sizes are reached, or if signs of reduced mobility, abnormal posture, abnormal gait, piloerection, weight loss of up to, but not reaching, 20% body weight or other indications of poor health occur (e.g. see Ullman-Cullere and Foltz [1999] 92 Lab. Animal Sci., 49, 319-323; a body score of 2 will be taken as a humane endpoint) the experiment will be ended and mice will be culled using a Schedule 1 method, unless the affected mouse or mice are clearly outliers. In such cases of outliers, only the affected mice will be culled if this can be done without reducing the mouse numbers to the extent that significant differences are no longer achievable, with culling of the remaining mice using a Schedule 1 method at the experimental endpoint.

We do not expect the subcutaneous or mammary fat pad tumour models that we will use in our planned studies to lead to metastases and we therefore do not expect complications due to metastasis. For pharmacokinetic, biodistribution, imaging and therapy studies, blood sampling will follow published guidelines (e.g. First Report of the BVA/FRAME/RSPCA/UFAW Joint Working Group on Refinement [1993] Laboratory Animals, 27, 1-12; www.nc3rs.org/3rs-resources/blood-sampling), with the exception of the pharmacokinetic studies, for which we need to extract 12-16 blood samples over a period of up to 14 days to monitor the clearance of the antibody/ADC from the blood. For these experiments, we remove 10 µl blood per sample, and the total blood volume will not exceed the limits set by the NC3Rs guidelines. Although we are using inbred strains of

mice, there is some mouse-to- mouse variability in pharmacokinetic assays. Hence the ability to longitudinally measure the same mouse improves data quality and reduces the numbers of mice needed. We typically do not see adverse events in the mice due to serial bleeds, but nevertheless will monitor the mice carefully.

We have also developed engineered antibodies that can be used to remove background of circulating, labelled antibodies or ADCs during whole body imaging, and these may be included in imaging studies. These agents function by either inhibiting the function of FcRn or pulling target-specific antibodies into degradative compartments in cells through receptor-mediated uptake. These specific antibodies are currently being used in Phase 3 clinical trials to treat autoimmunity, and we have also used them previously in whole body imaging studies to improve contrast. Based on these earlier studies/clinical trials, we do not expect to see adverse events.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our facility is very active disseminating any news, policy changes and/or advances in the 3Rs. This is accomplished by regular meetings (approximate 3 times per year) and email updates between the personal licence holders (PILh), project licence holders (PPLh), named animal care and welfare officers, animal technicians, home office liaison and compliance officer, named veterinary surgeon (NVS) and establishment licence holder. Any issues and incidents that PILhs need to be aware of are actively discussed when required.

Our facility is also requesting that PPLh and PILh undergo regular refresher training regularly to ensure that they remain updated of changes to best practices and aware of their responsibilities under ASPA and this is diligently recorded. Further to this, we also receive emails and discuss information from ASRU, NC3Rs and other organisations and details of training opportunities are provided to enhance welfare and research practice. We also encourage the members of our team to ensure animal welfare to the highest standard and to regularly learn the most recent advances of the 3Rs as shown in detail in NC3Rs (https://www.nc3rs.org.uk/) and NORECOPA (https://norecopa.no/alternatives/).

We actively review our procedures and if unnecessary harms are identified and can be reduced, modified protocols are implemented after discussion with researchers involved, PPLh, the NACWOs, and named vet as required. We also actively discuss this with our research group, peers in meetings, seminars, conferences to ensure that we are aware of any new or updated best practices.

67. Zebrafish models for understanding the genetics of neuromuscular 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

zebrafish, congenital muscular dystrophies, neuromuscular disorders, drug screen, genome editing

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 determine the genetic cause of conditions that affect the control and function of muscles (collectively known as neuromuscular disorders) using zebrafish.

To investigate the role of neuromuscular-related genes during embryonic development.

To advance our understanding of the mechanisms controlling muscle and motor neuron formation. To develop therapeutic interventions to treat patients with neuromuscular-related disorders.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these

could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

This project hopes to identify the involvement of new genes, signalling pathways and potential therapeutic targets for the treatment of patients that present with neuromuscular disorders from birth.

Patients with muscle related disorders are investigated for changes in their DNA which we suspect are disease causing. The zebrafish offers a model to validate whether these changes are causing the patient disorder. This can then be fed back to the patient forming part of the genetic diagnosis, essential for family planning and disease management.

To understand the role of these genes in development it is necessary to study both loss and gain of function and identify changes in signaling pathways and developmental processes. Altered pathways will be interrogated in zebrafish models, at the molecular level, for potential therapeutic interventions that will hopefully lead to effective treatments for patients.

What outputs do you think you will see at the end of this project?

We will publish in high impact journals and present our findings at relevant international conferences, providing important data for other laboratories that seek to eradicate muscle disease.

We will also disseminate findings of this research through teaching and student projects.

This project is highly interdisciplinary and will bring together experts from several fields including genetics/molecular biology, chemistry, proteomics, drug discovery, medicine, and developmental/cell biology. This work will bridge academic and industrial science, forging new collaborations for the generation of novel drug therapies

Who or what will benefit from these outputs, and how?

Reagents and tools generated by this project will benefit the entire field of muscle research. Probes and chemicals developed to detect and manipulate pathways affected in muscle related disorders will inform research and clinical communities.

Approximately, 50% of myopathy patients (people suffering from loss of muscle function) do not have an identifiable genetic cause. This project hopes to establish if novel genetic variants identified through whole genome and exome sequencing of patients with muscle related disease are disease causing.

This project will improve genetic counselling and informed choice.

Chemical screens performed on mutant zebrafish embryos will help identify therapeutics to sustain muscle tone and structure. This work will ultimately, in the long-term, improve patient quality of life and prognosis by providing drug treatments.

How will you look to maximise the outputs of this work?

This project will generate transgenic lines and mutant strains which be shared amongst the research community on request.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 10,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

This project will use zebrafish for research because they have many benefits that make them a great model for studying genetic diseases in humans. They are especially useful because they develop outside of the female and their eggs are transparent, which allows researchers to observe embryo development without harming the female. Zebrafish also produce many eggs, which means we can collect a lot of data without using too many animals. We will mainly use zebrafish embryos that are less than five days old, which means we will need fewer animals to answer fundamental questions about neuromuscular disease, than a similar study would using mice. They also share 84% disease causing genes in common with humans, meaning they offer an amenable animal model with similarities to human development and genetics. This project will also share resources with the zebrafish community to reduce the number of newly bred genetically modified fish.

Typically, what will be done to an animal used in your project?

This project forms part of a genetic diagnosis pipeline to identify changes in human patient's DNA which may be causing NMD. After we have identified candidate genes suspected of causing a disease, we then endeavour to provide evidence that they are functionally relevant. Typically, we will do this by creating specific NMD related mutant zebrafish and analyse the consequence of these genetic alterations on nerve and muscle development. To create mutant zebrafish, we inject a mix of RNA and protein into early embryos. This causes DNA breaks which are incorrectly fixed by innate repair mechanisms, which leads to mutations. We grow embryos up to adulthood (4- 6months) and identify the mutations by taking small biological samples from their tail, which grows back within 7 days and does not appear to cause a physiological stress response. The initial injections of DNA may lead to a low incidence of developmental defects. However, due to the recessive nature of the diseases we are working on, most embryos will develop normally and will be unhindered by a genetic change in one version (allele) of the gene of

interest. Similarly, we might want to look at where a gene product is localised over time, we can do this by creating fish with fluorescently tagged proteins. This is done by injecting DNA into an early-stage embryo (within first 30mins of fertilisation) with a special enzyme that causes random integration of the DNA into the fish genome. Fish are then grown up to adulthood and bred to find offspring that have inherited the fluorescent protein. With the transparent nature of zebrafish embryos, this process allows us to observe gene expression and protein localisation in live embryos, using a microscope.

In both cases, individual fish will be monitored on a daily basis for any adverse behaviours and euthanised via schedule one procedures, if necessary. Fish are culled at 18 months, as this is when they stop producing eggs and age-related disorders (scoliosis/lordosis) start to be observed.

What are the expected impacts and/or adverse effects for the animals during your project?

In the vast majority of cases, no adverse effects are expected through the normal maintenance of lines of adult fish. In some cases, genetic mutations may lead to minor body morphology or pigmentation changes – these are not expected to significantly affect viability or health. In a small minority of cases, the mutations or transgenes carried by the growing fish may lead to degenerative or behavioural alterations. It is difficult to predict in advance how often we will observe these phenotypes, but it is likely to be in less than 50 fish per year. If these unexpected phenotypes are potentially of interest with respect to our research goals, then we would examine the phenotype in more detail. This would be through post-mortem analysis of tissues of culled fish. Subsequent to phenotypic analysis (or if no phenotypic analysis is required) all fish displaying harmful phenotypes will be culled by schedule 1 methods.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The procedures proposed in this application use established standardised protocols. Injections of biological material are performed in embryonic stages (recently fertilised egg). The short-term effects of the injection will not cause pain or suffering. In most cases the embryos will develop to adulthood without any noticeable impact on health. However, it is possible that some embryos develop symptoms similar to the clinical features of the disease they are modelling. For example, when both genes inherited from their parents are carrying a mutation (change in the DNA) that affects gene function or when one inherited gene carrying a change is enough to affect embryonic development. Most stable mutant/transgenic lines will not have such adverse effects. Thus, in the majority of cases the procedure

will be classified as sub-threshold but occasionally individuals will be observed with a moderate severity classification.

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?

Embryonic development is a four-dimensional process (i.e., varying in space and time), and studying it therefore requires the analysis of whole developing embryos. Direct genetic studies of embryonic humans are difficult practically, and only descriptive analysis is possible, with experiments ruled out on ethical grounds. Tissue culture systems, although they can provide useful information on certain molecular or cellular phenomena, cannot mimic the complexity of functioning organs, let alone the developing embryo. Computer simulations can be valuable in extending theoretical approaches to embryonic development, but cannot tell us about those occurring in the embryo.

The zebrafish offers a fantastic whole organism system to study vertebrate development. Their embryos develop outside the female and are transparent allowing cell movements to be visualised using a microscope. This also means that the female does not have to be culled to collect the embryos. They produce large numbers of eggs, allowing many experimental individuals to be analysed.

Furthermore, they share over 84% of disease-causing genes in common with humans. Thus, a suitable replacement for higher vertebrate organisms.

Which non-animal alternatives did you consider for use in this project?

To investigate alternative non-animal methods for studying neuromuscular disorders, I searched Google and NCBI PubMed using the term “alternative models to study neuromuscular disorders” and found some suggested alternatives:

- Organ-on-a-chip (a bioengineered microdivice that could mimic neuromuscular junctions), which could be used to test the effects of compounds with a measurable readout such as voltage change. This technology is still in development, and the types of questions are still limited.

-2D in-vitro generation of motor neurons or skeletal muscle from patient derived induced pluripotent stem cells (iPSC). It remains unclear how accurate these mimic the matured physiology of MN and difficult to combine the two into a complementary system. Indeed, cells behave very differently in a 2D environment compared with a whole organism

- 3D in vitro models such as organoids and spheroids. The environment of the organoids/spheroids cannot replicate the multiple pathways, cell-types and chemical cues that cells respond to and interact with inside the organisms. Thus, the complete complexity of a living organism cannot be replicated in vitro.

- Computational. We already use a number of programmes to predict the likelihood a patient variant is disease. However, these are only predictive and do not interrogate the molecular mechanisms of disease. For that, we need biological samples.

Why were they not suitable?

Cells behave very differently in vitro compared to a whole organism and it is very difficult to analyse the repercussions of genetic changes in patients to how a cell behaves. Thus, muscle development and movement defects directly correlating to the patient clinical features can only be analysed in another living vertebrate 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 generation of transgenic and mutant zebrafish lines requires growing genetically modified (GM) fish to adulthood. In order to maintain each GM line, on average, 100 new fry per line will enter the aquarium every six months. This predicts 200 new fry per line per year, which is 1000 over the 5-year project. It’s anticipated that ten lines will be maintained under this project license, equating to 10,000 zebrafish.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Much of the proposed work will be performed at embryonic stages of development, up to but not exceeding 5-day post fertilization (dpf). Zebrafish are covered by the ASPA from 5dpf, using this approach we will therefore be reducing the number of animals being recorded under the act. With this in mind, we will be minimizing the potential for actual stress/pain by refining our experiments to embryonic stages of development.

Animal experiments will be designed efficiently using the most appropriate breeding schemes in order to produce as many embryos with the required genotype as possible, and therefore reducing the needless generation of non-affected animals. With this in mind, we will employ the NC3Rs' experimental design guidance and experimental design assistant (EDA) to plan our experimental

design, practical steps and statistical analysis utilising the advice and support for randomisation and blinding, sample size calculations and appropriate statistical analysis methods. We will use the EDA diagram and report outputs to support experimental planning with animal users.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Participate in the zebrafish community by sharing resources to reduce the number of newly bred genetically modified fish.

Experimental optimisation for drug screens and mutations will be performed and limited to embryonic stages of development.

Transgenic lines will use fluorescent labelled proteins to screen for genetic integration of DNA, increasing the chances of germline transmission and reducing needless rearing of experimental samples.

By optimising non-invasive genotyping of 3dpf embryos, this ensures only GA fish are grown to adulthood. Reducing needless animal usage in research.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Zebrafish produce hundreds of eggs per adult female, providing large datasets for genetic and statistical analyses. In our project, we focus on zebrafish embryos up to 5 days post-fertilization (dpf), which is when they start feeding independently. At this stage, zebrafish are protected under the Animals (Scientific Procedures) Act 1986. The use of zebrafish at earlier developmental stages reduces the number of animals needed compared to similar studies with mice, minimizing potential stress and pain. Zebrafish are also advantageous because of their small size and transparency, allowing non-invasive techniques. Unlike

mice, where drugs are typically injected, we can administer pharmacological agents directly into the fish embryo medium.

We will produce genetic mutations in potential disease causing genes using the CRISPR/Cas system, this offers several advantages over other techniques. Mainly, it is very easy to design CRISPR/Cas9 gene targeting constructs, the reagents are relatively cheap and easily accessible worldwide, the system is very efficient and can rapidly provide a phenotype in embryonic stages of development. This reduces the need to develop germline stable mutant lines which would otherwise require more animals.

Why can’t you use animals that are less sentient?

Much of the proposed work will utilise embryonic stages of development to answer fundamental questions regarding neuromuscular development. However, genetically modified animals grown to adulthood will be necessary to generate mutant and transgenic lines, that can later be analysed at more sentient stages. Zebrafish themselves are considered a relatively sentient vertebrate but are evolutionary more relevant to human disease than invertebrate models such as fruit flies or nematode worms, which are often used for genetic or developmental studies. Zebrafish have a complex genome, with many genes and regulatory elements that are similar to those in humans. Indeed, they share 84% disease causing genes with humans. This genetic complexity makes zebrafish a valuable model system for studying the genetics of human diseases and for testing potential treatments. Zebrafish embryos develop rapidly and are transparent, allowing researchers to observe and study developmental processes in real-time. This provides insights into how complex processes such as organ formation, cell specialisation, and control of cellular distribution occurs during embryonic development. This is not permissive in cell culture or indeed higher vertebrates such as mice.

Zebrafish embryos are easy to manipulate genetically, using techniques such as CRISPR/Cas9 gene editing. Coupled with their fast and prolific reproduction, we are able to rapidly and efficiently study the effects of specific genetic mutations (observed in patient cohorts) on neuromuscular development and function. Thus, the zebrafish offers an unrivalled rapid, clinically relevant, in-vivo system to analyse the repercussions of genetic variants found in patients with neuromuscular disorders.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Zebrafish are sensitive to environmental conditions such as water quality, temperature, and lighting. We will optimize these conditions to minimize stress and improve the welfare of the animals. For example, providing appropriate water filtration and maintenance protocols can reduce the risk of waterborne pathogens and ensure optimal water quality for breeding and welfare.

We will develop non-invasive techniques to collect data from zebrafish, reducing the need for invasive procedures that can cause pain and distress. For example, using non-invasive

imaging techniques to monitor movement. We will seek to implement and establish non-invasive methods to genotype adult zebrafish such as skin swabbing or extracting genetic material during embryonic stages of development.

We will continually seek to refine experimental procedures to minimize the harm to zebrafish. For example, using smaller sample sizes, less invasive methods of anaesthesia and analgesia, and minimizing the duration of procedures to reduce stress and pain.

We will use humane endpoints that define the point at which an animal is euthanized due to pain or distress. These endpoints will minimize suffering while ensuring that the scientific objectives are met.

We will continue share knowledge and resources to improve zebrafish welfare and refine procedures. For example, sharing information on best practices for breeding and maintaining zebrafish colonies, or sharing information on successful protocols for anaesthesia and analgesia. This will help improve animal welfare across the scientific community.

We will adopt Home Office (ASRU) published guidelines for efficient breeding of Genetically Altered Animals, this assessment framework aims to reduce animal wastage, cost and improve project outputs.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

To ensure experiments are conducted in a refined way in order to reduce waste, increase reproducibility and promote the 3Rs, we will follow published best practice guidance set out by international experts, called 'PREPARE' (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence). Some examples below of best practice strategies that we will use to ensure a culture of care in our research:

- Before conducting an experiment, we will review relevant literature to ensure that the experiment has not already been conducted and to gain an understanding of current best practices and standards in the field.

- We will create a detailed experimental plan that outlines the purpose of the experiment, the research question, the methodology, and the data analysis procedures. The plan will identify potential sources of error and outline steps to minimize them.

- We will select an appropriate research design based on the research question and the available resources.

- We will use standardized protocols for data collection and analysis to ensure that the results are reliable and valid.

- We will ensure that experiments meet ethical guidelines and standards, such as animal husbandry and welfare, humane end-point, and appropriate training has been provided to staff.

- We will conduct pilot studies to test the feasibility and reliability of the experimental procedures before conducting the full-scale experiment.

- We will use appropriate statistical methods to analyse the data and draw meaningful conclusions from the results.

- We will document all aspects of the experiment and report the results in a clear and concise manner using appropriate scientific reporting standards. This includes transparently reporting negative results or results that did not meet the expectations of the hypothesis.

-We will make sure that the descriptions of our published scientific methodologies are provided in sufficient detail for others to scrutinise and reproduce. We will use the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines to ensure thorough reporting is adhered to.

-We will use good practices set out by FELASA (Federation of European Laboratory Animal Science Associations) to ensure effective pain management is monitored to reduce suffering.

Overall, We will ensure that experiments are conducted with scientific rigor, transparency, and ethical considerations, to ensure the reliability and validity of our findings.

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 (NACWO, NTCO, Vet) and animal technicians at our establishment to review current approaches and whether more suitable protocols can be adopted.

We will attend NC3Rs events and workshops specific to zebrafish use, to keep UpToDate with the latest 3Rs advances and approaches. We will also attend relevant 3Rs webinars, institutional symposia, and international/national Zebrafish meetings to hear about novel 3Rs approaches, tools and technologies being used by other zebrafish researchers.

We will regularly check information on NC3Rs website, we have signed up to the NC3Rs newsletter, we will attend Regional 3Rs symposia.

68. Collection of Body Fluids and/or Tissues

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Body fluids, Blood, Tissues, In vivo, In vitro

Animal types	Life stages
Mice	adult, juvenile
Rats	adult, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall objective is to provide scientists with animal body fluids and/or tissues (typically blood samples) of appropriate quality to support 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?

This work is important for the calibration/ validation of processes that aim to analyse the composition and quality of a new medicine. This will ultimately result in the development of new medicines to treat people that are unwell, improve their quality of life and, in some cases, save their lives.

This work will also allow the development of in vitro tests that may replace the use of live animals, reducing the total number of animals required.

This licence will also support the validation of animal models of human disease.

What outputs do you think you will see at the end of this project?

This project is expected to result in:

- Development and validation of in vitro assays necessary for drug development. • Support of validated in vitro assays.
- Calibration or validation of technical instrumentation. • Quality assurance checks of experimental methods.
- Collating separate requests for supply of fresh body fluids and tissue ensures efficient use of animals e.g blood and liver could be supplied from 1 animal.

Who or what will benefit from these outputs, and how?

The development and validation of new assays is necessary for the efficient testing of new medicines for human use, which will treat or ameliorate human diseases benefiting patients.

How will you look to maximise the outputs of this work?

When terminal sampling is required, the collection of tissues will be maximised wherever possible, via an internal tissue user distribution list, so where possible tissues for multiple users can be obtained from the same animal. High quality -samples are the key criteria for ensuring successful outputs.

Quality control of ex-vivo assays will be performed, and results fed back to the project licence holder and others as appropriate, to determine if improvements to methods or processes are required.

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.

Mice and rats are widely used and studied in academia and industry so there is extensive knowledge of their physiology and they are known to share many genetic, anatomical, and metabolic characteristics with humans which are essential to set up assays relevant to the human clinical disease conditions. These species are therefore predominantly used for the development of new medicines and for that reason will be the species used for tissue and body fluids collection.

Wild type mice and rats will be used for the majority of the studies, however, animals with an altered immune system may be used to support programs of work where there is a clear scientific justification. It is not possible to say which genetically altered animal models will be required to provide samples but the adverse effects of the altered animal will be up

to mild severity.

For the scope of this project, adult animals will be mainly used unless other life stages are required. Juvenile life stages may be required for studies on human diseases that are age dependent, for example, studies using Non-obese Diabetic (NOD) mice can require the use of juvenile animals before they develop the immune and diabetic phenotype.

Typically, what will be done to an animal used in your project?

Samples from rodents will be taken under terminal anaesthesia and can include for example blood, cerebral spinal fluid, tissues and body fluids. Animals will be immediately euthanized after the collection of samples, without recovery from anaesthesia.

What are the expected impacts and/or adverse effects for the animals during your project?

No adverse effects are expected from procedures performed as all the procedures will be conducted under general anaesthesia and the animals will not recover.

It is not possible to say which GAA models will be required for sample provision, but the adverse effects of the altered phenotype of a GA animal will be up to mild 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)?

Over the course of this 5-year project we expect to use:
2000 mice & 500 rats, the severity classification will be non-recovery as all procedures will be conducted under terminal general anaesthesia.

In rare occasions, a GAA animal that has undergone a mild procedure on another licence may be returned on this licence (e.g. genotyping).

The adverse effects of an altered phenotype of a GA animal will be up to 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?

Studies requiring animal body fluids or tissues are only performed when all suitable in silico and in vitro alternatives have been exhausted.

The aim of this project is the validation and calibration of assays, development of new

analytical approaches and development of in vitro assays that can replace the use of live animals. For these, the use of freshly collected and high-quality samples such as whole blood or tissues are required as there aren't any biomaterials that could serve as substitutes due the complexity of constituents and biological properties of fresh samples.

Which non-animal alternatives did you consider for use in this project?

There are potential alternatives to animal tissues/ fluids such as culture cells, synthetic materials, in silico and DMPK modelling or the use of human patients' tissues and cells including the human organ- on-chip solutions.

Computing simulation and artificial intelligence tools to obtain rodent-human comparisons and to profile of compounds could, and will be, in some cases be used.

Wherever possible and practicable and where tissues are viable and available, studies will be performed on human tissues/fluids or on human or animal cell lines before animals will be used to provide samples for analysis.

Why were they not suitable?

Whilst the use of cell lines and computing modelling are being used as a first stage to demonstrate the viability of the studies or as a screening step before moving to rodent samples, these pose a limitation for some applications, some of the most common examples are:

- The validation of news assays frequently requires the use of control animal tissues for calibrations and to establish baseline parameters.
- The human organs–on-chips still requires powered validation and demonstration that a specific organ chip model can generate human-relevant results in a reproducible and statistically robust manner.
- The use of computational modelling and the development and validation of predictive models is limited by the dearth of biological data available. and frequently requires data from animal samples to refine gating algorithms. The phenotyping and validation of a genetically modified model of a human disease requires tissues and biofluids from these specific animals and cannot be replaced by any alternatives.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers of animals are an estimation based of the number of animals used on the previous licence where approximately 1800 mice and 200 rats were used. We expect to support a similar number of requests.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

A statistician sits on all UK-based Protocol Review Fora for in vivo studies and will have the opportunity to identify areas where statistical considerations may be valuable to study design.

Most of the work performed under this Project Licence does not involve formal statistical hypothesis testing, therefore there will be no requirement for the common statistical methods which support hypothesis testing - including formal power calculation (number of animals) or some design principles. The number of animals will normally be dependent on the volume of the blood or tissue type required for calibration purposes.

In vitro studies will be designed to ensure the minimum amount of tissues/ volume of body fluids are required providing the objectives of the study are achieved.

Scientists requesting the body fluid or tissue samples are always asked by the PLH to provide evidence of statistical advice received prior to work being undertaken.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

This licence coordinates the supply of tissues and body fluids and where terminal sampling is required, the sharing of tissues will be maximized using an internal tissue user distribution list or stored in a tissue bank so that, whenever possible, "stocks" of blood and tissues will be shared by different teams allowing the best use of each animal and reduce of the total number of animals required.

The licence also utilizes animals that become available as a result of the company's breeding strategy or rarely from cancelled projects.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Wild type and naïve animals will be used for the majority of the studies, however, immunocompromised animals can be used to support programs of work where there is a clear scientific justification. It is not possible to say which GAA models will be required for sample provision, but they are most likely to be mice with knock-out of murine receptors and knock-in of human proteins for example. The adverse effects of the altered phenotype of a GA animal will be of mild severity.

Non aversive handling of the animals using mouse/ rat tunnels or hand scooping will be used when manipulating animals.

Handling animals during the acclimatization period are being implemented across the animal unit to minimize anxiety.

All the procedures will be conducted on unconscious animals under general anaesthesia.

Why can’t you use animals that are less sentient?

All the procedures covered by this license will be performed under terminal general anaesthesia (non- recovery).

Other life stages or less sentient species such as prokaryotes or invertebrates won't be able to provide the amount or type of samples necessary for the scope of the projects supported.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All the procedures will be conducted under terminal anaesthesia to avoid any pain, suffering, distress or lasting harm. However, ongoing consideration of refinements throughout the lifetime of the licence for each individual procedure will be performed to minimize any stress and anxiety experienced by the animals prior to the procedures. Some refinements in place include:

- Rats are handled during an acclimation period to get familiar with the staff and less anxious when manipulated,
- Non aversive handling using mouse/ rat tunnels or hand scooping will be used when manipulating animals.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

- For blood collection, including routes, volumes and frequency:
- NC3Rs (National Centre for the Replacement, Refinement & Reduction of Animals in Research)- blood sampling general principles Website (https://www.nc3rs.org.uk/3rs-resources/blood- sampling/blood-sampling-general-principles),
- 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
- For monitoring of anaesthesia:
- NC3Rs (National Centre for the Replacement, Refinement & Reduction of Animals in Research) recommendations and guidance on anaesthesia and analgesia Website (https://www.nc3rs.org.uk/3rs-resources/anaesthesia)

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

A literature search for 3Rs including non-animal alternatives will be routinely conducted across at least two databases and website focused on NAMs such as Norecopa, NC3Rs and Center for Alternatives for Animal Testing (CAAT) and set up regular notifications for new publications relevant to this project. Whenever possible the PLH will attend courses and conferences (virtually or in person) focused on animal welfare and 3Rs.

69.Interventional procedures to improve the treatment of brain tumours

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

Key words

Brain Cancers, Glioblastoma Multiforme, Translational models, Novel Therapies

Animal types	Life stages
Sheep	adult, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The first aim of this project is to evaluate the safety of a novel device developed for specifically treating brain tumours in human 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?

Glioblastoma multiforme (GBM) is the most common form of malignant brain tumour in adults with an annual incidence rate of 3.19 per 100,000 population. The disease constitutes almost half of all malignant central nervous system tumours and 80%of all primary malignant central nervous system tumours. Despite our ever-increasing understanding of the disease processes underlying the biology of these tumours, glioblastoma remains an incurable disease with high mortality. Treatment typically consists of surgery followed by radiotherapy and chemotherapy (temozolomide). Over the last 20

years only minor improvements in 2 and 3 year survival rates have been achieved. Recent studies have shown that 2 year survival has increased from 9 to 18% and for 3 year survival it has increased from 4 to 11%. Five year survival rates unfortunately have remained largely unchanged at around 4%. Novel treatment strategies are required if we are to improve survival rates and quality of life for patients suffering from this devastating disease.

What outputs do you think you will see at the end of this project?

There is no cure for glioblastoma and the median life expectancy is 14 months. Electric field therapy is a new cancer treatment applied after tumour resection that directly interferes with cell division, slowing tumour recurrence and prolonging life. In randomised clinical trials alongside existing treatment, it increased median glioblastoma patient survival from 16 to 21 months. However, the single marketed device for glioblastoma applies electric field therapy externally from scalp pads across the whole head and must be worn for at least 18 hours a day. Patient compliance with using the device to get its maximal effect can be poor. A novel approach to improve treatment regimes is to deliver electric field therapy and focally, directly to residual cancer cells in the surrounding brain. To achieve this a unique, patented soft gel electrode is injected into the surgical cavity immediately after tumour resection.

Initially we will evaluate the performance of the device implanted into normal brains of healthy sheep. We will assess the devices' effects on animal health and on local tissue responses. This research will provide important data for future larger scale animal brain tumour model studies and eventually human clinical trials.

In order to maximise the outputs of this work the results will be published in high impact peer-reviewed oncology journals and presented at international conferences. Moreover, the outputs of this study aid finalisation of the surgical protocol for subsequent first in human trials as well as feeding into the GLP studies required for regulatory approval.

Who or what will benefit from these outputs, and how?

Fundamental aims to our initial project will be to provide in vivo data on the functionality and lifetime of the implantable electrotherapy device whilst assessing the effects on the animal and localised tissues. The short term impact of these results will help refinements to be made to the device and will provide important data for future larger scale animal studies and eventually human clinical trials. Estimates have indicated that an implanted electric field therapy device that functions permanently for the remainder of a patient's life could increase 5-year survival rates from 3% to 29%. This would be the most significant advancement made in the treatment of brains tumours for the last 20 years.

How will you look to maximise the outputs of this work?

This project is multidisciplinary and involves veterinary and medical colleagues from a number of scientific institutes. The Institute primarily involved in this research programme is a world class research institute with a track record of delivering cutting-edge translational research. It is the UK's foremost centre for livestock genetics and genomics and a world leader in methodologies for analysing animal and pathogen genomes. The Institute undertakes basic and translational science to tackle pressing issues in animal health and welfare, their implications for human health and the role of animals in the food chain. This project will also make use of large animal research and imaging facility which is fully supported and maintained by dedicated animal services and expert technical

personnel.

Our collaborating institute is recognised internationally as a research leader for clinical brain sciences. Research spans the laboratory and the clinic, combining molecular, epidemiology and clinical investigation to guide rationale innovation to improve patient care. Their research has led innovation in surgical simulation training around the world.

The first aim of this project will utilise cadaver experiments to refine the surgical procedure. These cadaver experiments will be essential to decrease the number of animals enrolled onto the actual studies. The study animals themselves will also provide us with multiple sources of information including, surgical, imaging and clinical (clinical examination, haematology and biochemistry) data. All animals will undergo a post-mortem examination at the end of the study for sample collection, these samples will be utilised to assess cellular changes from the device and biocompatibility. Animals will also act as their own controls for biocompatibility assessment where a section of non-implanted brain will be obtained for analysis. The aim of these initial experiments will be to publish our results including the surgical procedures, clinical data, imaging scans and histological analysis.

A commercial company involved in the research and development of medical devices has provided funding for this project. This funding will drive the translational aspects of the model forward.

Species and numbers of animals expected to be used.

- Sheep: 12

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

All animal experimentation raises ethical concerns. The past two decades have seen a considerable rise in the use of sheep to model human neurological disorders. While each animal model has its merits, sheep have many advantages over small animal models when it comes to brain research. In particular, adult sheep brains, as opposed to smaller commonly used laboratory animals, are more comparable in size and anatomical structure to the human brain. Their size also allows surgical procedures and imaging to be performed as would be performed in human patients. They also have much longer life spans, are amenable to regular sampling (e.g. blood) and are docile animals, making them useful for a wide range of in vivo studies.

Typically, what will be done to an animal used in your project?

The initial experiments will focus on pre-clinical testing of the electric field therapy device in normal healthy sheep. We will take an incremental approach starting with cadaver experiments to optimise the surgical procedures. These procedures will include craniotomy (surgical removal of a small part of the skull to expose the brain), removal of a small area of brain tissue, cranioplasty (surgical repair of the bone defect in the skull), gel and electrode placement and device/battery placement. Once optimised we will begin in vivo implantation and electrode stimulation experiments.

For the live animal experiments, we will require 2 groups of animals. The control group will undergo surgery for craniotomy and brain resection only, whereas the treatment group will undergo surgery for craniotomy (surgical removal of a small part of the skull to expose the brain), removal of a small area of brain tissue, cranioplasty (surgical repair of the bone defect in the skull), gel and electrode placement and device/battery placement. Supportive procedures during anaesthesia and surgery which may be carried out include: obtaining venous access, urinary bladder catherisation, rumen trocharisation and performing an electroencephalogram.

Animals will arrive at the institution for a period of acclimatisation. A typical protocol will include:

Day -7: Sheep will be blood sampled for baseline evaluation of organ function (haematology/biochemistry) and undergo clinical and neurological examination. Sheep in the treatment group will also be fitted with a ram harness which will house the battery pack after surgery.

Day 0: Sheep will be anaesthetised for surgery and electroencephalogram (EEG). We anticipate surgery will last for around 1-2 hours. Following recovery from surgery sheep will be provided with painkillers (analgesics) and antibiotics and monitored for signs of post-operative complications.

Day 3: Sheep will be anaesthetised for MRI and EEG. In the treatment group this MRI will be regarded as a go/no go point. If imaging identifies problems with the implanted device that will prevent stimulation being carried out for the remainder of the study the animal may be euthanised under anaesthesia. Any euthanised animals will undergo post-mortem examination and brain tissue collection.

Day 7: For animals in the treatment group the device will be switched on. The device remains on for the duration of the study. Battery changes or charging and electrode checks will occur daily.

Day 56: Sheep will undergo terminal anaesthesia for MRI, EEG and post-mortem examination with brain tissue collection.

All sheep will be blood sampled and undergo clinical and neurological examination every 7 days post- surgery.

We will only progress into performing surgeries for device implantation and stimulation once 30 days have elapsed from the sheep having surgery without device implantation in order to assess any medium-term complications that might occur from the surgery itself. All sheep will receive peri-and post-operative analgesics and antibiotics.

What are the expected impacts and/or adverse effects for the animals during your project?

Pre-clinical testing of the electric field therapy device. We expect the animals undergoing anaesthesia, blood sample collection, imaging and surgery may experience moderate severity. Stress for repeated restraint for blood sampling and neurological examination will be reduced through a period of acclimatisation.

The majority of the potential adverse effects from this set of experiments will occur from the surgery itself and the development of a surgical site infection. During surgery if there is

significant haemorrhage which prevents completion of the procedure or would likely prevent normal anaesthetic recovery the animal will be euthanised without recovery from anesthesia. Post-operatively, neurological signs related to complications would include (but not linmited to) cranial osteomyelitis, meningitis, abscess formation, ataxia and/or seizures, abnormal reflexes, proprioception, impaired vision, change in behaviour, including increased aggression or increased/decreased sensitivity to sensory stimuli.

These potential adverse effects are most likely to occur immediately or within the first 7-10 days following surgery. Animals will receive peri-and post-operative antibiotics and painkillers (analgesics) to reduce pain and likelihood of infections. The risks of sheep developing signs of progressive disease will be mitigated with daily monitoring of the animals with clinical and neurological examinations at least once a week.

Animals showing severe neurological signs unlikely to improve with treatment will be euthanised immediately. Moderate neurological signs in which sheep are still able to eat and drink but do not show normal behaviour e.g. ability to walk around the pen and interact with pen mates, which fail to improve/resolve with treatment within 7 days will be euthanised by a schedule 1 method followed by post-mortem examination and collection of brain samples for histological examination.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

100% of the animals will be expected to experience moderate severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Novel cancer treatment strategies require validation in translational models before progressing into human clinical trials. Sheep are now regarded as an excellent model for brain research and can provide us with an opportunity to gain invaluable information on the safety and efficacy of novel treatment strategies. This important data can be used to strengthen applications for human clinical trials.

Which non-animal alternatives did you consider for use in this project?

There are no non-animal alternatives that can provide medium to long term safety data on the use of the electric field therapy device for the treatment of glioblastoma.

Why were they not suitable?

There are no non-animal alternatives that can provide medium to long term safety data on the use of the electric field therapy device for the treatment of glioblastoma.

Safety data from our experiments will include results from the surgical procedure itself, biocompatibility and the foreign body response (the capability of an implanted device in the body to exist in harmony with tissue without causing damaging effects) and systemic effects. The use of in vitro cell line studies may give an indication of acute cellular toxicity from the materials used in the construction of the implanted device but are unable to accurately assess biocompatibility issues and systemic effects. Cell line studies lack the complex tissue microenvironment, stromal and immune cells that are required to fully assess biocompatibility and safety. These models are also unable to be successfully utilised for our studies that require advanced imaging and surgery.

Brain tissue explants can overcome some of the issues related to in vitro cell line experiments, however culture can only be maintained for relatively short periods of time and again cannot account for the animal's immune response and tissue microenvironment which are likely essential factors involved in the foreign body response in the live animal.

Safety data can also take months to become fully evaluated, these time frames cannot be simulated in a laboratory setting.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The first aim of this project is to evaluate the safety of a novel device developed for specifically treating brain tumours in human patients. As these first experiments are proof-of-concept pilot studies, with no pre-existing data, it is not possible at this stage to carry out power calculations for the subsequent time course experiments. Data acquired from the pilot studies will be used to design appropriately powered, larger-scale time course studies. Indicative numbers of sheep are included for the protocols; however, these numbers may change slightly depending on pilot results. In every case, only the minimum number of animals necessary to achieve the study objectives (clearly defined a priori) will be used.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The first aim of this project will utilise cadaver experiments first to refine the surgical procedure. Cadavers will most likely come from sheep that have been euthanised at the end of an unrelated study. These steps will include craniotomy, partial brain excision, cranioplasty, gel and electrode implantation and device/battery placement.

These cadaver experiments will be essential to decrease the number of animals enrolled onto the actual studies. In terms of biocompatibility the device is constructed from well characterised biocompatible materials. The study animals themselves will also provide us

with multiple sources of information including, surgical, imaging and clinical (clinical examination, haematology and biochemistry) data. All animals will undergo a post-mortem examination at the end of the study for sample collection, these samples will be utilised to assess cellular changes from the device and biocompatibility. Animals will also act as their own controls for biocompatibility assessment where a section of non-implanted brain will be obtained for analysis.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

This project is designed to generate novel essential results regarding the safety of novel brain treatments and will produce a new ovine brain tumour model for translational research.

To enable us to achieve our aims we are utilising methods which our group is extremely knowledgeable in, have been previously validated in our labs and have publications to support for work. We will use these methods to generate novel results which have previously never been reported in the literature. The methods we shall use include:

- Ex vivo cadaver brain tissue experiments Brain surgery in sheep
- Clinical care of sheep having undergone a brain procedure
- Histological assessment of implantable medical devices

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 initial experiments will focus on pre-clinical testing of the electric field therapy device in normal healthy sheep. These experiments are based upon a surgical model which we will develop. To ensure the model is appropriate and refined we will take an incremental approach starting with cadaver experiments to optimise the surgical procedures. These procedures will include craniotomy, partial brain excision, cranioplasty, gel and electrode placement and device/battery placement. Once optimised we will begin in vivo implantation and electrode stimulation experiments.

For the live animal experiments, the control group will undergo surgery for craniotomy and brain resection only, whereas the treatment group will undergo surgery for craniotomy, partial brain excision, cranioplasty, gel and electrode placement and device/battery placement.

Animals will arrive at the institution for a period of acclimatisation. Harness and jacket acclimatisation will be performed prior to surgery to ensure they are comfortable with harness and jacket.

We will only progress into performing surgeries for device implantation and stimulation once 30 days have elapsed from the sheep having surgery without device implantation in order to assess any medium-term complications that might occur from the surgery itself.

All animals will receive peri-operative and post-operative antibiotics and analgesics to reduce pain and likelihood of infections. The risks of sheep developing signs of neurological disease will be mitigated with daily monitoring of the animals and with clinical and neurological examinations at least once a week.

Why can’t you use animals that are less sentient?

It is necessary to use animals in this project in order to meet the primary objectives because there are no alternative systems that offer the ability to assess the safety of the treatment device over a prolonged period of time. Performing experiments on sheep will therefore provide the most comprehensive translational data set which can be used to support applications for future human clinical trials.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We aim to minimise any stress or discomfort to experimental animals. Sheep will be housed in groups of more than 2 with respect to their treatment.

The initial experiments will focus on pre-clinical testing of the electric field therapy device in normal healthy sheep. We will take an incremental approach starting with cadaver experiments to optimise the surgical procedures. These procedures will include craniotomy, partial brain excision, gel and electrode placement and device/battery placement. Once optimised we will begin in vivo implantation and electrode stimulation experiments.

We will only progress into performing surgeries for device implantation and stimulation once 30 days have elapsed from the sheep having surgery without device implantation in order to assess any medium-term complications that might occur from the surgery itself.

In the treatment group the post-operative day 3 MRI will be regarded as a go/no go point. If imaging identifies problems with the implanted device that will prevent stimulation being carried out for the remainder of the study the animal may be euthanised under anaesthesia. Any euthanised animals will undergo post-mortem examination and brain tissue collection.

All sheep will receive peri-operatively analgesics, as part of a refined multimodal anaesthetic protocoll, which will be continued for as long as needed in the post-operative period. Sheep will undergo daily checks and weekly clinical and neurological examination. Animals with any signs of distress or disease will be examined by a vet and treated as appropriate or euthanised. Humane endpoints will be based on moderate severity limits, with consideration of weight loss, body condition, appetite, respiration, behaviour and dehydration according to a clinical/neurological scoring system.

Collaborators in the USA have conducted preliminary experiments involving device implantation with no stimulation (n=1) and device implantation with stimulation (n=1). Post-operative monitoring was conducted up to 28 days post-implantation. These studies have allowed some refinement of the surgical and post-mortem procedures. During the 28 days post-implantation results have indicated that none of the animals showed clinical sign of

disease and were unremarkable on clinical examination immediately before euthanasia for the termination of the study. Although these affected animals have the potential to develop neurological signs these results so far indicate that the procedures and monitoring protocols are mitigating the risks.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We have used the PREPARE guidelines for planning the animal experiments. To enable us to achieve our aims we are utilising methods which our group is extremely knowledgeable in and have been previously validated in our labs. We will use these methods to generate novel results which have previously never been reported in the literature. The methods we shall use and have previously published upon include:

- Ex vivo cadaver brain tissue experiments Brain surgery in sheep
- Clinical care of sheep having undergone a brain procedure
- Histological assessment of implantable medical devices

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

All group members will regularly keep updated about 3R advances largely through online courses and research.

Online resources will include: Pubmed and Google searches, Altweb (global clearinghouse for information on the 3Rs; http://altweb.jhsph.edu/resources/links.html), Norecopa (https://norecopa.no/3r-guide-database), National Centre for the 3Rs (www.nc3rs.org.uk/informationportal), Alt.Tox (advancing non-animal methods of toxicity testing through online discussion and information exchange; http://alttox.org), AnimAlt-ZEBET (https://www.bfr.bund.de/en/zebet_database_on_alternatives_to_animal_experiments_on_the_internet__animalt_zebet_-1508.html), DB-ALM ECVAM Database Service on Alternative Methods to Animal Experimentation (https://ecvam-dbalm.jrc.ec.europa.eu/), Fund for the Replacement of Animals in Medical Experiments (http://www.frame.org.uk)

Regular contact with the veterinary services at our Institutes will provide first contact for any 3R updates. Local courses run through these institutes will also be attended.

70. Investigation into drug and vaccine delivery

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

Drug, vaccine, medicine, sex, fed or fasted

Animal types	Life stages
Mice	juvenile, adult, aged
Rats	juvenile, adult, aged
Hamsters (Syrian) (Mesocricetus auratus)	juvenile, adult, aged
Hamsters (Chinese) (Cricetulus griseus)	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 develop new medicines and vaccines, and to investigate the factors which influence the in vivo fate of a medicine or vaccine, and the resulting biological responses.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Many existing medicines and vaccines are not 100% effective. In addition, for many diseases, there are no commercially-available medicines or vaccines. This work will generate new knowledge which can, in the near future, be used to produce medicines and vaccines which are more effective, less toxic, cheaper and more acceptable to people.

What outputs do you think you will see at the end of this project?

The primary expected benefit is the publication of new scientific knowledge about how:

- factors, such as age, sex, the fed/fasted status of an individual,
- the medicine/vaccine preparation
- influence the in vivo fate of medicines and vaccines and the biological responses.

Who or what will benefit from these outputs, and how?

Throughout the life of this project, data produced will be presented at national and international conferences and published in academic journals. The information will provide a new understanding of how the preparation of a medicine/vaccine and the attributes of the user such as a person's sex, age and fasted/fed status influence the medicine's/vaccine's stability, in vivo fate, and the biological responses elicited.
In the medium term, other researchers and the pharmaceutical industry will be interested in our data and our medicine and vaccine preparations.

In the long term, our data may have implications for the conduct of animal experiments. For example, experimenting on fasted and fed animals, males and females and young and aged animals may become routine, in order to identify potential confounding factors early on in drug and vaccine development. Ultimately, patients will benefit from new and/or improved medicines and vaccines.

How will you look to maximise the outputs of this work?

Subject to the requirements for patent protection, we will make our findings available to other scientists through publication in open-access journals and presentations at scientific conferences and meetings. Data will be deposited in repositories such as figshare, and will also be made available to other researchers via a link in the related publications.

We will take as many tissues as possible at post-mortem, and will make such tissues available to other researchers we collaborate with.

Species and numbers of animals expected to be used.

- Mice: 1824
- Rats: 1872
- Hamsters (Syrian) (Mesocricetus auratus): 168
- Hamsters (Chinese) (Cricetulus griseus): 168

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Animals will be used as it is not possible to measure ‘what the body does to a medicine/vaccine’ and ‘what a medicine/vaccine does to the body’ without the ‘body’, in this case, the body of an animal.

Mice, rats and hamsters will be used as their biological systems and physiological samples are similar to those of humans. Collection of these animals' faeces, urine, blood, saliva, vaginal secretions, which are similar to their human equivalents, will enable us to measure the medicines'/vaccines' biological responses and in vivo fates. It would not be possible to obtain such samples from 'lower' species, such as invertebrates (e.g. nematode worms) or immature forms of vertebrates such as larval forms of zebrafish or single-celled organisms such as yeast, which do not produce similar physiological samples.

Juvenile, adult and older adult life stages will be used to investigate the influence of age on 'what the body does to a medicine/vaccine' and 'what a medicine/vaccine does to the body'. It is important to determine these influences to ensure medicines and vaccines are effective in all age groups, and if they are not, to take appropriate action, for example, to increase/reduce the amount of medicine/vaccine given.

Typically, what will be done to an animal used in your project?

When animals arrive at the Animal Facility, they will be given time (one week) to get used to their new environment before experimentation. Animals will be housed in groups (for social animals) or singly (for solitary ones) in cages. In some instances, they may be housed individually in specialised cages so that their food and water intake can be closely monitored and/or controlled, and urine and/or faeces can be easily collected. They may be weighed and marked by ear clipping. Water will be freely provided, but food may be withheld from some animals for a limited duration, to investigate the influence of fasting. A different type of food may be given to some animals to determine the influence of food type. Blood, urine, faeces, saliva and vaginal fluids may be collected at weekly intervals.

In some experiments, blood and faeces will be collected several times a day. Animals may have a thin tube inserted into a vein when blood needs to be collected several times a day, and in such cases, animals will be housed singly if needed. Some animals may be shaved and/or tape-stripped over a small area of their skin, to enhance drug/vaccine permeation into the skin and/or to measure drug/vaccine levels in the skin. Tape-stripping involves the placement of an appropriate adhesive tape on shaved skin, followed by application of light pressure by placement of the operator's finger on the tape for durations up to 30 seconds, followed by removal of the tape. Tape-stripping may be repeated a maximum of 15 times.

Animals will be given a medicine/vaccine/placebo preparation by up to 2 of the following routes: injected in a muscle or vein or abdomen or skin, or injected under the skin, or applied on the skin surface, or placed under the tongue or in the mouth or stomach or nose or anus or vagina. In some cases, the drug/vaccine/placebo may be included in the animal's diet or drinking water. Prior to the application of a preparation on the skin, a small area of the skin may be shaved. On rare occasions, animals will receive a medicine or vaccine preparation by up to 3 of these routes.

The administration of a preparation may be repeated up to 7 times, for example, at weekly or more frequent, such as daily, intervals. Control animals may not receive any preparation. Animals may undergo imaging. Local or general anaesthesia may be used as required prior to the administration of substances and imaging to minimise any pain or discomfort. At the end of the experiment, animals will be killed. The maximum duration of experiments will be 6 months. The longer duration will enable us to monitor the long-term effects of our medicine and vaccine preparations, and their in vivo fates.

What are the expected impacts and/or adverse effects for the animals during your

project?

Animals will experience no lasting harm but may experience mild to moderate and transient discomfort and pain from:

- being weighed and marked,
- being anaesthetised,
- fasting or change of diet for a limited duration,
- administration of substances by injection in a muscle or vein or abdomen or skin, or injection under the skin, or application on the skin surface, or placing under the tongue or in the mouth or stomach or nose or anus or vagina.
- collection of blood, urine, faeces, saliva, vaginal fluids and skin strips.

Animals that are cannulated (to allow repeated blood collection per day) may experience greater discomfort and pain (for example, if the cannulation site becomes bruised, swollen or infected, or if there is blood loss or if the cannula becomes blocked, over a longer duration of several hours.

Older animals may experience age-related conditions such as obesity, lameness, reluctance to move (possibly due to arthritis onset), presence of visible lumps (possibly due to tumour formation). We will monitor such adverse effects by assessing the clinical state and weight of the animal and keep records using the Severity Scoring Sheet. Aging can also lead to changes in how a drug is handled by the body (which might lead to increased toxicity of chemicals), changes in the immune system, e.g., the immune system is less competent. To reduce possible adverse events, such as greater toxicity of a drug/vaccine/carrier due to aging, pilot studies in a few aged animals will be conducted for drugs/vaccines/carriers with limited toxicity profiles and dose studies in aged 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)?
Mice - Approximately 90% of animals are likely to experience moderate levels of severity. The remaining 10% of animals are likely to experience mild severity.
Rats - Approximately 90% of animals are likely to experience moderate levels of severity. The remaining 10% of animals are likely to experience mild severity.
Hamsters - Approximately 90% of animals are likely to experience moderate levels of severity. The remaining 10% of animals are likely to experience mild severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Our aim is to develop new and more efficacious medicines and vaccines. We need to

prepare and optimise our preparations and test whether they are effective. Animals are necessary to determine the fate of medicines and vaccines, and the biological responses they cause.

Animals will be used as it is not possible to measure ‘what a body does to a medicine/vaccine’ and ‘what a medicine/vaccine does to a body’ without the ‘body’, in this case, the body of an animal.

Which non-animal alternatives did you consider for use in this project?

We have considered non-animal alternatives, such as cell culture, tissues taken from freshly killed animals and synthetic materials such as synthetic skin and simulated salivary, gastric and intestinal fluids.

A range of in vitro and ex vivo experiments using these non-animal alternatives will be used to optimise drug and vaccine formulations prior to the in vivo work. For example, vaccine formulations will be characterised with respect to properties such as their stability using simulated salivary, gastric and intestinal fluids. In vitro immune cell assays will be used to give an indication of the potency of vaccine formulations and select the ones most likely to be effective. These in vitro and ex vivo experiments will partially replace animals and lead to a reduction in the number of animals that would be necessary. In silico studies will also be considered when appropriate.

Why were they not suitable?

The non-animal alternatives cannot fully replace animals. Synthetic materials such as synthetic skin and simulated salivary, gastric and intestinal fluids are not exact replicas of physiological samples. In addition, cell culture, tissues taken from freshly killed animals and synthetic materials are isolated systems and are not in contact with the rest of a body. Thus, communication among the different body systems - which leads to the ultimate biological response from a medicine / vaccine - cannot take place. As a result, the non-animal alternatives do not give the full and true picture of a medicine's or vaccine's effects.

It has also been reported that the in vitro-in vivo extrapolation from cell cultures is not yet satisfactory enough for cell culture to replace animal experiments. Similarly, other researchers showed the current limitations of in vitro and in silico methods in an in vitro – in silico - in vivo drug absorption model, where the methodologies employed influenced the relevant outcomes. Thus, animals cannot be fully eliminated from this project.

In some cases, it may be feasible to use in vitro cell culture studies to select the most promising components of vaccines, when a range of these is available. The cell culture assays will be used to choose the best candidates to go forward in animal experiments. Thus, fewer animal experiments would need to be conducted.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g., pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

An estimation has been made based on the research questions to be answered, the number of experiments and the number of animals per experiment that is feasible over the 5-year period.

The experimental design will take into account previous experience (ours, our collaborators and from the literature) of similar research whenever this is available. Pilot studies with 2-3 animals will be used when needed, for example, to ascertain the shortest duration of anaesthesia needed, or that the desired targeting (e.g. to a specific site in the gastro-intestinal tract, such as the large intestine) has been achieved. Factorial design will be used where appropriate to reduce the number of animals. The Experimental Design Assistant (at the NC3Rs website) will be used to design experiments and to calculate sample sizes. Animals assigned to experimental groups will be randomised and operators will be blinded wherever possible. In some cases, it will not be possible to blind operators, such as, when the drug/vaccine formulations are designed to be different, for example, when a gel is being compared to a liquid as drug/vaccine carriers. Experiments will be powered to use the minimal number of animals required to produce significant data. Power calculations, previous research and/or pilot studies will be used to determine sample size which will produce the desired power. During the design of each experiment, the nature of the statistical tests to be used will be decided on and this will be revisited during data analysis. Should there be difficulties/need for statistical analyses that I am not familiar with, expert advice will be sought from statisticians. The appropriate parametric and non-parametric statistical analyses – for each experiment - will be conducted. The Experimental Design Assistant at the NC3R website will be consulted for its suggested statistical analyses of our experiments.

Examples of statistical analyses to be conducted include t-tests (e.g. when 2 vaccine formulations or immune response at 2 mucosal sites) are compared; ANOVA (for more than 2 groups), Repeated measures ANOVA (when immune responses/drug levels generated by several formulations have been measured over time); Pearson product-moment correlation coefficient (e.g. to examine correlations between in vitro and in vivo immune responses). Non-parametric tests such as Mann- Whitney, Nemenyi's test, Spearman Rank Order Correlation will be conducted when required, e.g. when the data does not satisfy the requirements of parametric tests. I have used such tests over many years of experience in data analysis and in all my publications, data has been statistically analysed.

In publications, the experiments will be reported according to the ARRIVE guidelines. The numbers below are estimates of maximum numbers.

Protocol 1. Experiments to measure drug/vaccine/excipient/carrier levels in blood. Number of rats per group = 6

Number of animal groups = 6 (a selection from: young males; adult males; old males, young females, adult females; old females, different strains).

Animal feeding status = 2 (fed or fasted) Number of delivery vehicles = 4 Number of routes = 2

Total maximum number of rats= 6 x 6 x 2x 4x2 = 576 Some experiments will be conducted in mice

Number of mice = 6 animals per group x 2 groups (selection from male/female/different strains/ages) x 2 groups (fed/fasted) x 4 formulations = 96
Some experiments will be conducted in hamsters.

Number of hamsters = 6 animals per group x 2 groups (male/female) x 2 groups (fed/fasted) x 2 formulations = 48

Protocol 1. Experiments to measure drug/vaccine/excipient/carrier uptake in different tissues. Animals are dosed with drug/vaccine formulations, and drug/vaccine/carrier levels in tissues are measured at different time points following dosing. At each time point, animals are killed and tissues are taken to measure drug/vaccine/carrier levels.

Drug
Number of rats per group = 6
Number of animal groups = 6 (a selection of the following: young males; adult males; old males, young females, adult females; old females; different strains).
Animal feeding status = 2 (fed or fasted) Number of delivery vehicles = 2 Number of routes = 2
Number of time points = 4
Total maximum number of rats = 6 x 6 x 2 x 2 x 2 x 4 = 1152 Vaccine
Number of mice per group = 6
Number of animal groups = 4 (a selection of the following: young males; adult males; old males, young females, adult females; old females, different strains).
Number of delivery vehicles = 2 Number of routes = 3
Number of time points = 4
Total maximum number of animals = 6 x 4 x 2 x 3 x 4 = 576 Some experiments will be conducted in hamsters
Number of hamsters = 6 animals per group x 1 group x 2 delivery vehicles x 2 routes x 4 time points = 96
Protocol 2. Vaccine administration and measurement of immune responses Number of mice per group = 6
Number of animal groups = 8 (a selection from the following: young males; adult males; old males, young females, adult females; old females; different strains).
Number of formulations = 12 Number of routes = 2
Total maximum number of animals = 6 x 8 x 12 x 2 = 1152 Some experiments will be conducted in rats
Total maximum number of animals = 6 rats per group x 2 groups (male/female) x 4 delivery vehicles x 3 routes = 144
Some experiments will be conducted in hamsters
Number of hamsters = 6 animals per group x 8 groups x 2 delivery vehicles x 2 routes = 192 A summary of animal numbers is shown below

protocol	mice	rats	syrian hamsters	chinese hamsters
1	672	1728	72	72
2	1152	144	96	96
total	1824	1872	168	168

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Before conducting animal experiments, several medicine and vaccine preparations will be produced, thoroughly tested in the relevant non-animal alternatives and only the most promising will be used in animal experiments.

The NC3Rs' Experimental Design Assistant (EDA) will be used to plan the experimental design, practical steps and statistical analysis utilising the advice and support for randomisation and blinding, sample size calculations and appropriate statistical analysis methods. We will use the EDA diagram and report outputs to support experimental planning with animal users.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Collection of samples such as blood and faeces from each animal prior to administration of substances will allow each animal to act as its own control and will reduce the number of control animals needed. Collection of blood and faeces, which is a very simple procedure and causes minimal distress to animals, at regular (e.g. weekly) intervals during an experiment will enable timely analysis of biological responses as they develop, and the results will be used to decide, for example, whether to continue or terminate the experiment. This will ensure that experiments are not terminated prematurely, and thus will optimise the number of animals. Criteria for continuing or terminating the experiment will not be pre-set.

The use of both male and female animals in the same experimental group (e.g. 3 males and 3 females in a group) will reduce the total number of animals needed, as separate male and female groups (e.g. 6 males in one group, and 6 females in another group) will not be used. Our accumulated data over a large number of experiments which will indicate whether there are any variability between males and females, and this information will be useful to other researchers who may be encouraged to include both sexes in their experimental group. Thus, our project could lead to a reduction in the number of animals used by other researchers.

It will not always be appropriate to include both sexes in the same experimental group. For example, when the influence of sex is being investigated or when it is known that males and females respond differently or when medicines and vaccines are being developed specifically for one sex, e.g. maternal vaccines.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Wild-type mice, rats and hamsters will be used as their physiological samples, such as faeces, urine, blood, saliva are similar to their human equivalents, and the possibility of collecting such samples will enable us to measure the effects, such as immune responses

generated, of our formulations.

Methods and reagents to test the biological responses that medicines and vaccines cause in mice, rats and hamsters are also widely available, which means the animal experiments can yield useful data.

Animals will be housed mostly in groups due to their social nature, and will be housed singly, when appropriate. For example, adult Syrian hamsters, which are normally solitary, and cannulated animals (where the cannulation may be interfered with by other animals in the same cage) may be housed individually. Environmental enrichment will be added when appropriate.

The methods of substance administration and collection of samples from these animals have been optimised over decades of research and by researchers world-wide in order to be of short duration and to use anaesthesia when required, such that the most transient and least amount of pain, suffering and distress is caused to these animals. For example, when the oral route of drug/vaccine administration is being tested, we will give the formulation in the mouth in some animals, and by oral gavage in other groups. Placement of the formulation in the mouth is more refined than oral gavage and the data will be useful to ourselves and other researchers for future experiments. For example, similar responses following formulation placement in the mouth (buccal delivery) or in the stomach (oral gavage) can lead to the favouring of buccal delivery over oral gavage, ultimately refining oral delivery.

Why can’t you use animals that are less sentient?

Less sentient and ‘lower’ non-mammalian species, such as invertebrates (e.g. nematode worms) or immature forms of vertebrates such as larval forms of zebrafish or unicellular organisms such as yeast cannot be used as their biological systems are too different to those of humans to enable testing of our medicine / vaccine preparations and to provide relevant results. It will not be possible to collect physiological samples, such as faeces, urine, blood, vaginal secretions, saliva for analysis.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animal suffering will be minimised by appropriate observation, care and handling, and by the use of sufficient anaesthesia and analgesia when required. Mice and rats will be mostly kept in groups in cages as they are social animals, and when isolation is necessary, cage dividers or metabolic cages will be used. Adult hamsters, which are normally solitary, may be housed individually. Environmental enrichment will be added when appropriate.

Mice will be picked up by using a cupped hand and/or handling tunnel (rather than by the tail). Rats habituated to handling will be picked up by grasping them around the shoulders. Handling tunnels and/or cupped hands will be used to pick up hamsters.

When required, anaesthetics will be used briefly during administration of medicine / vaccine preparations to animals to reduce distress. For oral administration, the use of jelly will be investigated as a refinement to oral gavage. The use of micropipette-guided drug administration (MDA) method (as described in https://www.sciencedirect.com/science/article/pii/S0889159120302543?via%3Dihub), where the drug/vaccine is dissolved in a mixture of water and condensed milk, and animals

are trained to voluntarily consume the drug/vaccine formulation will be considered as this is less stressful to the animals than oral gavage.

Anaesthetics may also be used briefly during collection of certain animal secretions, such as saliva, and vaginal secretions, to reduce distress. The duration of anaesthesia will be determined in pilot studies with 2-3 animals, to achieve the shortest duration which enables collection of the secretions. Collection of animal faeces and urine will be conducted non-invasively by temporarily placing the animals in a cage and collecting the faeces and urine from the cage. Materials such as LabSand will be used for urine collection as this does not disturb the animals.

Collection of blood from animals will be conducted via a superficial vein such as the tail vein and animals may be warmed to dilate the blood vessels, while care is taken to avoid hyperthermia and dehydration. For repeat blood sampling in the same day, cannulation will be considered. Cannulated animals may be purchased or cannulation may be conducted in house.

Animal welfare score sheets will be used, and humane endpoints will be defined. Trained technicians in the Animal Facility House and the Biological Services Unit manager will also be consulted and will provide help during experiments, for example, with drug/vaccine administration and collection of physiological samples.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

PREPARE guidelines (http://journals.sagepub.com/doi/full/10.1177/0023677217724823),
ARRIVE guidelines 2.0 (https://arriveguidelines.org/arrive-guidelines),
LASA Current Publications (https://www.lasa.co.uk/current_publications/),
Good practice guidelines (e.g. such as ‘Refining Procedures for the administration of substances, https://pubmed.ncbi.nlm.nih.gov/11201285/)report of the BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement (2001) Laboratory Animals 35, 1-41’ , and the NC3Rs Resources such as papers and videos on animal handling and restraint, blood sampling, anaesthesia, analgesia, welfare assessment, administration of substances and experimental design will be used.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will periodically review the NC3Rs website. We have signed up to the NC3Rs newsletter, we will be in regular contact with the NC3Rs Regional Programme Manager, and attend Regional 3Rs symposia. We will also closely follow the research literature for advances in methodologies using animals and non-animal alternatives. We will discuss possible implementation of any advances in the 3Rs with animal technicians, veterinary surgeons and others responsible for animal welfare at the animal unit.

71. Modelling mitochondrial dysfunction in mice

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

Mitochondrial diseases, Phenotyping, Biomarkers, Experimental 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 is to generate novel mouse models of primary mitochondrial disease and to develop a comprehensive phenotyping platform for their characterisation. The mouse strains which exhibit faithful representations of human disease will then be used for pre-clinical testing of treatments for mitochondrial 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?

Mitochondria are tiny energy generators that exist in large numbers (100s–1000s) inside human cells. Primary mitochondrial diseases (PMDs) are a large group of genetic disorders that impair mitochondria and the body’s ability to make energy. They are among the most common inborn errors of metabolism, with a minimum incidence of 1:5,000 in the

human population. Advances in genomics have allowed for improved diagnosis of PMDs.

Mitochondrial diseases can cause disabling symptoms that may impact very severely on quality of life. Though individually rare, as a group PMDs are a common cause of brain and musculoskeletal diseases. The effects of faulty mitochondria seen in PMDs are also present in other common diseases, such as dementia, diabetes, and cancer. A better understanding of PMDs has the potential to help us understand these more common conditions much better.

PMDs are caused by changes to DNA inside the cell nucleus or in the small amount of DNA inside the mitochondria themselves (called (mtDNA). As mitochondria are present in almost all cells, any system in the body can be affected. Muscles are particularly susceptible to reduced energy, which causes fatigue and weakness.

PMDs can cause symptoms at birth and in early childhood which can be life- limiting.

PMDs during adulthood cause a wide variety of medical problems, depending on the body parts affected, and present both emotional and physical challenges for patients.

There are currently no effective cures or treatments for PMDs. A significant barrier to PMD research, and that of other diseases in which mitochondria play a role, is a lack of relevant mouse models.

Recreating PMDs in mice will allow researchers to confirm that specific genetic mutations cause PMD symptoms, find new ways to measure the disease over time, and develop and test new treatments that can then more safely progress to trials in humans.

It has been very challenging to create mtDNA models of human PMDs in mice, but new tools to change mtDNA have recently been established. Also, modern techniques are now available to measure mouse development, movement, and behaviour that can be applied over long periods without stressing mice. This will allow researchers to study PMDs in these animals more accurately and humanely.

The work carried out under this licence aims to: (1) deliver new insights, therapies and biomarkers of PMDs; and (2) help understand the role of mitochondria in other common diseases (e.g., dementia, diabetes and cancer).

What outputs do you think you will see at the end of this project?

Work conducted under this project license will generate data and new knowledge that will help advance our understanding of how mitochondrial diseases develop, and what changes at the level of the molecules and cells makes these diseases get worse over time.

We aim to generate new mouse models that develop symptoms of mitochondrial disease that are seen in patients in the clinic, and to develop highly sensitive methods to detect these symptoms. Our work will help advance knowledge of mitochondrial disease processes and identify potential new drugs or druggable targets. We also aim to identify new biomarkers of disease (e.g. proteins or DNA in the blood that are released from damaged tissues) that we could use to track disease progression.

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. We will also share the models that we generate. 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 mouse models and discoveries generated under this programme of work. This will be due to the development of new research tools or models, 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 biomarkers and new 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. We will also publish results where the mouse model does not recapitulate the human condition, thereby preventing unnecessary repetition of experiments.

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: 13000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mitochondrial diseases are observed across the lifespan, from birth to old age. Therefore, we will use animals across the life stages- neonatal, juvenile, adult and aged mice.

To study disease biology and ask how a protein effects the disease process we need to use genetically modified mice which do not express that protein or express modified (or mutant) forms of the protein that is found in patients. We can genetically modify the mouse genome to generate mammalian models in which to study the same biochemical changes seen in patients with mitochondrial diseases. We need to do these experiments in whole animals as the diseases we are studying often affect multiple organ systems within the body, and we need to be able to investigate the interplay between the different organs.

There are currently no human or mouse cell models (in vitro models) capable of a comprehensive study of the mitochondrial dysfunction across multiple, interacting physiological systems. We cannot use less sentient animals such as insects or fish as their nervous system does not work in the same way and they lack some of the immune cells present in humans, meaning that they cannot model the diseases that we are interested in

accurately. Additionally, the highly sophisticated research tools/reagents required to investigate disease mechanisms and translate directly to higher mammals are established.

Mice remain the model of choice due to the wealth of the genomics information available, their relevance to humans, and the relative ease of generating, establishing and cryopreserving mouse colonies. Moreover, well-developed and characterised inbred strains of mice present an opportunity for reducing variability and therefore enhancing reproducibility.

Typically, what will be done to an animal used in your project?

A significant number of the mice on this licence will be used only for breeding. This is due to the need to obtain a suitably sized cohort with the correct genetic alterations that can be studied. We will also maintain breeding lines to share with collaborators for use on other projects authorised to accept GA lines.

Initial characterisation of models will include a series of phenotyping tests to understand how the model develops with age. For example, mice will be assessed using home cage analysis for behaviour, motor and metabolic parameters, mice will also be tested outside the cage to look at other behaviour and motor phenotypes, they will have their sensory system assessed to check for hearing and sight issues, and blood taken for biochemical analysis. These test will be used to detect phenotypes that reflect human symptoms seen in the clinic. This set of tests will be repeated at multiple time points across the animal's lifespan (initially up to 15 months, then in aged animals).

Once the models are fully characterised, substances may be administered to attempt to modify some of the phenotypes. In these cases, mice may have repeated dosing, followed by assessment in a subset of phenotyping tests that we expect to see changes in.

Animals may undergo a combination of both substance administration combined with behavioural testing.

A small subset of lines will go through a short pipeline for studying sensorimotor function, visual and auditory function, and pain response to both noxious thermal and mechanical stimulation.

Experiments will vary in duration from days to months. All protocols will end by 26 months of age. Animals that are remain alive over 52 weeks (i.e. 12 months old) will have increased welfare checks to look for age-related phenotypic changes.

What are the expected impacts and/or adverse effects for the animals during your project?

Mitochondrial disease models may develop symptoms such as weight loss, muscle weakness, tremor or anaemia. We expect these symptoms will develop very slowly over the lifetime of the mouse. We will not allow these mice to age beyond 26 months.

In a subset of strains, we will be breeding the genetic alteration for the first time. It is possible that this will affect the viability of those mice in period between birth and weaning.

Some of the tests that we perform will require the mouse to be under anaesthesia.

Some mice may lose weight after repeated anaesthesia or may develop aversion to the

anaesthetic.

Expected severity categories and the 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 - 50% of all animals used in this project Mild - 10% of all animals used in this project Moderate - 40% of all animals used in this project.

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 are required for the study of mitochondrial diseases because they are highly complex disorders, often affect multiple organ systems within the body, and can result in different clinical phenotypes. For the majority of mouse models we use, we will be studying multiple systems involving many organs of the body, and the interplay between them (e.g. behaviour and metabolism). There is currently no human or mouse cell models (in vitro models) capable of a comprehensive study of the mitochondrial dysfunction across multiple, interacting physiological systems. We also want to test potential therapies to improve mitochondrial function. It is important that this is done in live animals to test the effects of medicines on the whole body and mice have the lowest neuro-physiological sensitivity. 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.

We have established a strong international network of collaborators over the years and have access to a rich source of information across many different fields in science as well as in husbandry and welfare. This network can be canvassed to explore areas of research that lie outside of our own expertise, to ensure that we are able to identify areas of work where either the mouse model or the phenotyping test is not appropriate to the scientific question.

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. We have accumulated archival tissue banks of frozen and formalin fixed tissues from our previous models and from human healthy and diseased human tissues. These samples are used in multiple ongoing projects to minimise the number of animal models used.

We routinely use human cells in culture (including cell lines from patients with mitochondrial disease) to understand and model the biological processes affected by

mitochondrial dysfunction cells and to perform initial drug testing prior to performing work in animals. We can also use special cell systems called induced pluripotent stem cells to make particular types of cells such as muscle or brain cells, and investigate the effect of mitochondrial dysfunction in those cells in culture.

We have started developing 3D culture systems as an alternative method for drug screening and to understand the effect of mitochondrial dysfunction cell metabolism, for example in the intestine. We are also using co-cultures of human neurons and inflammatory cells to model how mitochondrial dysfunction affects the interplay between different cell types. The use of these cell systems prior to work in animals means that we can refine the work we do in animals and minimise animal use.

Why were they not suitable?

Whilst they 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 recapitulate the behaviour of a drug in a complex biological system.

Whole-body work is needed to understand mitochondrial disease pathology, as there is evidence for the interplay of between different tissue/organ systems. For example, patients with a mitochondrial disease called Leigh syndrome have a defined type of brain abnormality. The mouse model recapitulates these lesions quite closely.

However, it has been shown that cell culture and brain organoid models do not recapitulate the types of brain abnormalities observed and do not respond to interventions that were successful in the mouse due to a lack of immune cells which are cells necessary to drive the disease, thus limiting their use in pre-clinical investigation. As organoid systems become more advanced, this may become possible, but technology is currently lacking for these applications.

Less sentient models have guided our selection for new and existing PMD models. For example, we have developed a novel Drosophila (i.e. fruit fly) mtDNA mutator strain to study cell-autonomous/non- autonomous effects of mitochondrial dysfunction. This has been used to study mitochondrial homeostasis and quality control in neurodegenerative diseases. However, this model cannot completely answer questions about the action of these genes in mammals nor their use as therapeutic targets. We aim recapitulate this fly model in a mutant mouse model and characterise the resulting mice using neurological, metabolic, and behavioural tests.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Breeding of mice with changes to their mitochondrial DNA (mtDNA) poses different challenges when compared to mice with changes to their nuclear DNA. For example; mtDNA is only inherited through the female line, and certain level of the mtDNA mutation (usually over 70%) is required before mice show mitochondrial dysfunction in their tissues and/or symptoms of mitochondrial disease. In addition, mtDNA randomly segregates into egg cells (oocytes) meaning that a female with 75% mutated mtDNA may produce individual egg cells (and therefore offspring) which contain anything from 40-80% mutated mtDNA. This means we may have to breed a larger number of animals than for nuclear mutations in order to select experimentally relevant mice carrying the required proportion of mutated mtDNA. We will avoid over supply as much as possible by carefully selecting breeding stock with high mutation levels to increase the chances of producing experimentally relevant mice.

The phenotyping pipeline has been designed in collaboration with many international renowned mouse centres and has involved the input of many laboratory animal experts.

By utilising this pipeline we maximise the data obtained from each cohort of mice.

Combining tests in the same mice allows the interpretation of data to be correlated directly, rather than inferred. This allows us to carry out more advanced statistical analysis to detect correlated trends between assays. Directly correlated effects have a greater sensitivity as the major source of variance (between mice) is removed, opening the possibility to detect subtle effects resulting from gene deletion, thus resulting in more data from fewer mice.

We have 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. Group sizes will be larger when we need to keep mice into old age (up to 26 months). This is because, in our experience there is significant attrition (especially in males) when co-housing animals to this time point. Attrition rates are different for different background strains, but we will use data from other ageing projects to ensure that the appropriate numbers for statistically and biologically relevant data will be available throughout the study.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The PREPARE guidelines 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. Careful planning of colony expansion will ensure any surplus of animals generated is kept to a minimum. This is a particular challenge in the breeding of mitochondrial DNA mutant mice; however, the members of the team are uniquely equipped to minimise waste as they have significant experience in breeding these strains.

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. If this is not possible then pilot studies are performed to reduce numbers and inform future studies going forward.

All breeding for experimental cohorts is performed according to precise breeding calculations which have been designed in consultation with the wider team who have extensive experience with both nuclear and mtDNA mutated lines. We will continually review data from our breeding colonies, and refine and update the breeding strategy if required. This is to ensure we produce the maximum number of animals that can be used in experimental procedures and minimise the number of mice which have the incorrect genotype.

Where breeding information is not known (i.e. for new lines) a small pilot breed will be carried out first to assess the viability of mice with a particular genetic alteration. In addition, we will work with our colony management team and follow local guidelines to preserve colony health, monitor any unexpected genetic defects and ensure preservation of the genetically altered lines.

All new GA lines that we generate and characterise will be will be shared with the wider research community upon request, via frozen sperm or embryos, depending on the strain.

Tissue is regularly shared between projects to maximise outputs from animal procedures and minimise numbers of animals used. The group have accumulated archival tissue banks of frozen and formalin fixed 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.

All of the disease models chosen are the lowest severity model that can be used to answer

our research questions. Mitochondrial dysfunction can affect multiple organs of the body; therefore to understand how mitochondrial disease develops we need to use genetically altered mice in which the mitochondria do not work properly.

Mitochondrial diseases progress over time in humans, with some symptoms not appearing until late in life; therefore we have to age the mice in order for those symptoms to develop.

We will continuously monitor the behaviour and condition of these mice, using non or minimally-invasive tests wherever possible to ensure minimal suffering. Where a mouse is undergoing multiple tests, these will be done in a specific order to limit cumulative suffering and allow the mice to overcome one test before they do the next test. Pain relief and anaesthesia will be given when required to limit pain, suffering and distress.

Why can't you use animals that are less sentient?

The mouse is the lowest mammalian species (in terms of the sensitivity of the nervous system) in which we can perform the full range of genetic manipulations that we need to understand how mitochondrial dysfunction affects multiple organ systems, the clinical phenotype of the mouse, and how we might improve mitochondrial function with therapies.

It is possible to carry out some manipulations in frogs and fish but the processes being studied here are 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. For example, mitochondrial disease is known to have 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 we want to model are not the same between these species and mammals therefore some of the disease mechanisms may not be the same.

In addition, 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.

Mice remain the model of choice due to the wealth of the genomics information available, their relevance to humans and the relative ease of generating, establishing and cryopreserving mouse colonies. Moreover, well-developed and characterised inbred strains of mice present an opportunity for reducing variation and therefore enhancing reproducibility.

The life stages used under each protocol have been carefully considered and meet the experimental life stage needed. For example, early interventions for mitochondrial diseases need to be given to very young mice as therapies may be more effective early in life. Older adults ("aged" animals) will be characterised to model how the disease progresses and to see if clinical symptoms emerge later in life, as this is what we see in human mitochondrial disease.

Less sentient models have guided our selection for new and existing PMD models. For example, we have developed a novel Drosophila (fruit fly) model, which has high levels of mtDNA mutations to study the mechanisms of mitochondrial quality control in neurodegenerative diseases. However, this model cannot completely answer questions about the action of these genes in mammals nor whether they are useful as targets for drug treatments. We aim model this GA fly in the mouse, and characterise the resulting GA strain.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

For all tests mice are only housed in modified cages or arenas for the minimum time needed to gather meaningful data. Mice undergoing phenotyping tests are monitored more frequently and are removed from tests if they appear to be suffering from an adverse stress reaction, or other unexpected adverse effects of the phenotyping tests. Mice which have had anaesthesia have extra monitoring until fully recovered and extra checks when back in the holding rooms. When general anaesthetics are necessary, the combinations with least adverse effects will be used, for example for all tests inhalation anaesthetics will be used, with the exceptions of the Auditory Brainstem Response, the Electroretinography and the Optical Coherence Tomography tests which cannot be carried out with the mouse on a face mask. Pain from tail bleeds is reduced by using local anaesthesia. Mice over 12 months of age will have increased welfare checks to look for age-related phenotypic changes.

We have modified handling techniques to reduce stress on animals.

Pipelines are designed with consideration given to the overall experience of the mouse and the number of type of tests any one animal will go through.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Routes and volumes for administration of substances are taken from LASA guidelines.

The animal house has full accreditation. To conform to these standards we must ensure a high level of quality control on all fronts including husbandry, phenotyping and administrative processes.

Standard operation procedures for most tests have been generated using data and expertise from multiple animal houses and can be found at https://www.mousephenotype.org/impress

The requirements of the ARRIVE guidelines will be considered at all times during the planning of the experiments and strictly adhered to when publishing the data.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Project leads will attend general 3Rs symposiums in the UK and abroad over the course of this project. From these we may gather information on refined phenotyping techniques or housing and husbandry methods. Moreover, it is our intention to continue to present any techniques that we develop ourselves in posters and papers in the relevant scientific/animal care forums.

More specifically, members of the phenotyping team will attend conferences that focus on all aspects of the project and the pipeline. Any new developments which could impact the studies will be discussed within the team of investigators and action taken if relevant to the development of the phenotyping pipeline.

72. Multi-modality sensorimotor integration in the developing Xenopus tadpole nervous system in health and injury

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Brainstem, Spinal cord, Motor control, Sensory modality, Injury

Animal types	Life stages
Xenopus laevis	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The project aims to investigate how frog tadpole brain and spinal cord integrate sensory signals to generate motor behaviour in normal conditions and in case of spinal cord injury.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Different types of sensory information including that for touch, light and water flow in aquatic animals are sent to the brainstem for integration before motor decisions are made. Once the motor decision is made, the spinal circuit is responsible for executing the motor commands. The exact structure of the brain circuits and the integration process are poorly understood. In case of spinal cord injury, mammalians have very limited capacity for repairment in contrast to the remarkable ability of some amphibians/reptiles to grow back amputated limbs or tails. We plan to use simple amphibian tadpoles to study their brainstem and spinal cord functions in normal conditions and how the spinal circuit recovers its function after injury. In-depth understanding of how the brainstem and spinal

cord carry out these sensory motor tasks are critical in the eventual design of potential new treatment for spinal cord injury and other motor disorders.

What outputs do you think you will see at the end of this project?

How sensory information is processed by the central nervous system to control motor behaviour remains a fundamental question in neuroscience research.

However, the study of sensory and motor functions in higher vertebrates including mammals has been hampered by their complexity and poor accessibility. This project will generate novel insights in the following areas:

How sensory information is processed depending on the brain state (rest or motion) to produce opposite motor outputs. Our data show activating tadpole sensory systems at rest starts swimming but the same stimulation during ongoing swimming stops the movement. We are going to reveal the neural mechanisms to explain how the motor decision are reached differently.

How sensory stimulation at varying intensities and durations result in different motor responses like flexions, turning, swimming and struggling. We expect different neural circuits to be recruited following varied sensory stimulation.

How the regenerated spinal cord after tail amputation mediate swimming. Will the regenerated spinal cord have similar anatomy and physiology to its intact counterpart?

The results will be written and published on peer-reviewed scientific journals.

Who or what will benefit from these outputs, and how?

The direct outputs from this project include new understanding on how the nervous system process sensory information and make motor outputs. In short and medium terms, the scientific community, especially mammalian researchers, will learn the general principles on sensory motor integration from our studies. In longer terms, our findings will be of interest to both medical practitioners and pharmaceutical companies to improve their understanding of how motor outcomes are made in normal and injury conditions, which could potentially help guide their design of treatments.

How will you look to maximise the outputs of this work?

The project will draw in new approaches from collaborators. For sensorimotor integration, we are going to collaborate with a colleague for two-photon imaging and analyses. For spinal cord injury studies, we are going to work with another colleague for molecular mechanisms underlying spinal cord repair. We are also going to work with colleagues at other establishments to generate new transgenic animals.

Results will be disseminated at conferences before formal publications. Finalised results will be published on mainstream neuroscience journals. Some key findings will also be listed on the lab websites to maximise exposure. Negative results will be published on journals like PlOS ONE so other researchers can avoid repeating the failed experiments.

Species and numbers of animals expected to be used.

- Xenopus laevis: 238

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Pre-feeding Xenopus tadpoles at two days old have a simple nervous system when ocular vision and hearing are not developed. Their sensory motor responses have been well characterised and there has been good understanding of the underlying neuronal circuits. Also, tadpoles do not have bones in early developmental stages, allowing high access for physiology studies.

Xenopus tadpoles possess the ability to regrow the tail after amputation.

Typically, what will be done to an animal used in your project?

A volume of up to 0.6 ml Human chorionic gonadotropin (hCG) will be injected into adult Xenopus dorsal lymph sac to induce mating so embryos can be collected to raise tadpoles. The injection procedure lasts 10 to 20 seconds, causing some transient pain to the animals.

Adult Xenopus may also be subject to general anaesthesia using Tricaine Methanesulfonate (MS222) once to facilitate microchipping.

The physiological experiments in this research programme are performed mostly on pre-feeding stages of Xenopus tadpoles, which are therefore not covered by the Act, or occasionally on tissue resulting from a schedule 1 procedure applied to later stage tadpoles. The physiology experiments normally last up to 4 hours, after which tadpoles are killed by fixation or Sch1 methods.

What are the expected impacts and/or adverse effects for the animals during your project?

Genetically altered animals are generated by licensed institutions and screened only to keep the lines with mild or little effects on animal health and behaviour.

A regulated procedure is needed to inject Human chorionic gonadotropin (HCG) into adult Xenopus dorsal lymph sac to induce natural mating so embryos can be collected. The injection procedure doesn’t require anaesthesia, only causes transient discomfort to the animals and doesn’t have long- lasting adverse effects. Therefore, the procedure is mild.

General anaesthesia is also considered mild with animals recovering fully within two hours.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severity level is mild and all animals should fall in this category.

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?

The study of neural circuit functions has to be carried out in situ where the innate connectivity is intact and animal behavioural outputs can be monitored.

Which non-animal alternatives did you consider for use in this project?

Cell culture: Searches have been carried out on https://pubmed.ncbi.nlm.nih.gov/ and google scholar using terms “Cell culture, locomotion, motor system”. None of the results has shown animal locomotion-like rhythms generated by the cultured cells.

Computer modelling: Searches have been carried out on https://pubmed.ncbi.nlm.nih.gov/ and google scholar using terms “modelling, locomotion, motor system”. Many results have demonstrated good reproduction of locomotion of the modelled animal species, though in most cases statements of the need for more biological data were made.

Organoids: Searches using “spinal organoids” and “brainstem organoids” on https://pubmed.ncbi.nlm.nih.gov/ and google scholar have returned results showing organoids raised from human pluripotent stem cells resemble some anatomical features of spinal cord and brainstem. However, the sensory systems are missing.

Why were they not suitable?

Cell culture loses all the innate connections between the nerve cells in intact preparations. Cell culture cannot produce network outputs bearing physiological significance.

Computer modelling is only powerful when it is based on physiology data, which has to be collected from animals first. With more biology data accumulated in this project, we will keep refining our existing computer model of tadpole motor circuits, which will gradually help to reduce animal use.

Organoid research is still at the very early stage of development. Similar to cell cultures, organoids do not yet have neuronal circuits matching the real animal nervous system.

Especially, the lack of sensory systems renders them unsuitable for studying sensorimotor integration.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise

numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We plan to use 238 adult frogs during the project. This is based on:
the continued use of the existing breeding colony of 64 wild type and 16 genetically altered frogs at the beginning of the project;
the projected replacement of 56 frogs reaching 10 years of age during the project; c) on average, replacement of 14 frogs with poor embryo qualities or poor health per year;
d) the addition of 32 new genetically altered frogs during the project.

A breeding colony of 64 to 112 frogs (depending on embryo quality and experimental needs) is needed to achieve successful breeding twice a week after hormone injection procedures into 2 to 4 pairs of frogs, on a 3-5 month rotatory basis, to provide a steady supply of tadpoles throughout the project. The total hormone injection procedures will be 1500 over 5 years, averaging 300 per year.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The physiological experiments in this research programme are performed mostly on pre-feeding stages of Xenopus, which are not covered by the Act, or occasionally on tissue resulting from a schedule 1 procedure applied to later stage tadpoles.

The main data we gather are physiological recordings, the success of which varies considerably from experiment to experiment and from experimenter to experimenter.

The mean and standard deviation of most parameters are unknown. Therefore, it is technically difficult to use power calculations and experimental designs to pre-determine the number of tadpoles needed for each subset of experiments. Although the experiments on pre-feeding tadpoles are not regulated, more efficient data gathering from tadpoles can reduce the number of protocols used for inducing mating of adult Xenopus. Based on previous comparable physiology projects, we will aim for sample sizes of 8 to 10 tadpoles for each set of experiment.

We have the following measures to make the best use of tadpoles successfully raised from each injection and reduce the number of adult animals and the regulated procedure outlined in protocol 1:

Because the mild Human chorionic gonadotropin injection procedure is to induce natural mating, we can reuse the animals many times after their recovery period. The number of animals can be reduced significantly by selecting proven breeders for reuse.

Embryos are raised at different temperatures so from one injection there are normally tadpoles for use on 3 to 5 consecutive days.

Once sufficient biological data have been gathered in this project the information can be incorporated into computer models.

By combining physiology with imaging and molecular biology tools, the amount of experimental data we can collect from each tadpole will be maximised.

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 the following ways to reduce the number of animals required and the number of procedures:

We can reuse the animals many times after their resting periods of three to six months because the mild Human chorionic gonadotropin injection procedure is to induce natural mating. The number of animals can be reduced significantly by selection of good breeders for re-use for up to 10 years.

The use of mutant and transgenic Xenopus laevis as soon as they become available, in collaboration with colleagues from other institutions.

Embryos are raised at different temperatures to stagger their development so from one injection there are normally tadpoles for use for three days.

Some experiments difficult to carry out in live tadpoles will be run in computer modelling once essential biology data have been collected.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The physiology studies are carried out on pre-feeding Xenopus tadpoles, which are considered insentient.

To obtain embryos to raise tadpoles, regular HCG injections into adult Xenopus dorsal lymph sac are needed to induce mating. The injections of fluid up to 0.6ml using a 25G needle only causes transient pain with the frogs recovering minutes afterwards without leaving any change to animal behaviour or health. This procedure is considered mild.

Why can’t you use animals that are less sentient?

Xenopus tadpoles starts to feed when they are four days old. Tadpoles of most of the experiments on this project are going to at pre-feeding stages, which are considered insentient.

Some tadpoles older than four days (up to 15 days old) will be used for the spinal cord regeneration project. These tadpoles will be terminally anaesthetised first. Then the circulatory system was disrupted via removal of the heart, and subsequently the forebrain was also removed before physiology experiments.

How will you refine the procedures you're using to minimise the welfare costs

(harms) for the animals?

The pre-feeding tadpole themselves are insentient but we need to breed them using adult frogs. The procedure of subcutaneous injection of human chorionic gonadotropin (hCG) had been refined from the initial injection into the leg muscles to injections into the dorsal lymph sac. The procedure previously consisted of priming injections followed by final injections a few hours later. Now the procedure has been simplified to one full dose injection.

We also have the following tested methods to minimise frog welfare costs in animal husbandry:

Regular handling of the same animals to reduce fear/stress.

Add artificial plants and hiding tunnels to frog holding tanks to enrich their environment.

Adding pig hearts as food supplement.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The experiments are conducted on pre-feeding tadpoles, which are considered insentient. For the procedure on adult animals, we follow:
Smith AJ, Clutton RE, Lilley E, Hansen KEA, Brattelid T. PREPARE: guidelines for planning animal research and testing. Lab Anim. 2018a.
https://doi.org/10.1177/0023677217724823.

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.
For adult animal care, we mainly follow the two published guidance:
Xenopus tropicalis and Xenopus laevis environmental parameter standard operating procedure (Kroll lab,
https://www.xenbase.org/xenbase/jsp/common/showWiki.jsp?Protocols)
NXR publication: Husbandry, general care, and transportation of Xenopus laevis. Sean McNamara, Marcin Wlizla, and Marko E. Horb. Methods Mol Biol. 2018 ; 1865: 1–17. doi:10.1007/978-1-4939- 8784-9_1.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will stayed informed about 3Rs advances by checking NC3Rs website regularly (https://www.nc3rs.org.uk/) and attending some relevant events, paying attention to animal husbandry literatures, communications with other colleagues working in the same field and also by our establishment NIO. Any advance that can lead to 3Rs will be taken into account of in the following experimental design. For example, we are going to introduce new transgenic lines in our experiments as soon as they become available.

73. Polyclonal antibody production for biological research

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Antibodies, Medical research, Biological research

Animal types	Life stages
Sheep	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project is to continue the production of antibodies in sheep. These antibodies will be used in biological and medical research across a wide range of applications.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 proteins that are made in response to a vaccine (although in the "real world" they are made in response to exposure to an infection like a cold or the ‘flu). Antibodies are able to very accurately identify specific proteins. For example, during the Covid pandemic, lateral flow devices that were used to test for Covid infection, used an antibody that identified a specific part of the virus and indicate whether someone was

infected. Antibodies are therefore absolutely essential to nearly all types of research on living things, including medical research into the causes and treatment of disease. Over the past 5 years, I have generated antibodies to many hundreds of proteins that are important in a range of research questions, primarily in medical research. These antibodies have enabled new information to be gained aout the protein of interest and have either validated a target for further work, such as drug development for a disease, or shown the target to be not important. This later result is equally important when conducting research to reduce time and money wasted on invalid targets.

I, and many other researchers, are keen to move away from animal use in research but we have not found an alternative approach that provides the necessary specificity to proteins of interest across the range of applications needed such as immuno-blotting, immuno-precipitation, immuno-fluorescence and histochemistry procedures. Only a live animal possess the intact and functional immune system required to produce highly specific and high affinity antibodies. This licence will enable me to continue to produce antibodies for my own research, the research of my colleagues and the research of others across the globe where it can be justified there is both a good reason and no alternative to animal produced antibody. This licence will help accelerate the research of all these researchers for many hundreds of targets in medical research and other important areas of study.

What outputs do you think you will see at the end of this project?

The primary output from this project will be products that will be used to generate new knowledge. The antibodies produced under this project will be used in a vast array of areas. This new knowledge may span cancer to brain disease, viral infections to veterinary disease, food production to weed control.

New knowledge on alternative approaches to antibody production in animals will also be gained.

Antibodies will be used in internal research as well as being made available to the wider research community via our reagents distribution service. Knowledge will normally be disseminated in the form of scientific publications, presentations and posters. Antibodies will also be used in collaborations with the pharmaceutical industry to help develop new therapeutic targets, where the output will be treatments for disease.

On my previous licence, I generated the first complete set of antibodies for the study and diagnosis of Covid-19. We made 42 antibodies to teh SARS-CoV-19 virus as well as other related viruses such as MERS and SARS. This work enabled several key publications, a new therapeutic target strategy and critically, access to key Covid reagents to the global research community. During the period of this proposed licence, I aim to do the same for Influenza A and B, a global killer and major economic burden, to accelerate research and treatment for this disease. Other examples of the work I will undertake on my proposed licence are ;
The development of a new set of antibodies to SARM1 and proteins on the SARM1 pathway. I am working with a major pharmaceutical company to discover new targeting strategies to the horrific motor neuron family of disease (MNDs). SARM1 is directly implicated in the cause of MNDs these antibodies will enable the study of how and why neurones die as the disease develops. Partnering with a major pharmaceutical in this work will increase the chances of the research being translated to treatments.

The production of a set of antibodies against receptors in heart tissue that we have found may be implicated in accelerating heart muscle death during reperfusion of oxygen

following a heart attack. We will use the antibodies to detect the expression of these receptors in a cell type know as cardio myocyctes that we are able to grow into mini hearts in a dish. We use these organoids to study what happens to heart muscle during heart attack and work out which receptors may be useful in helping treat damaged hearts after heart attack.

We are working with a pharmaceutical company to create a model human lung in cell culture to help us study how a devastating disease termed fibrosis develops. We have found multiple previously unstudied proteins that we think may be involved in disease. During this proposed licence, we produce antibodies to these proteins to help us understand their role in disease.

Who or what will benefit from these outputs, and how?

The immediate research group of the project licence holder. The research Unit and institute. Other academic researchers globally. The pharmaceutical industry. Food production companies. Veterinary scientists. More long term as a result of this, veterinary practitioners and their patients. Clinicians and their patients.

How will you look to maximise the outputs of this work?

One major may is to offer the ability to rapidly produce antibodies as a service to other researchers in a not for profit approach. Extensive collaboration with the pharmaceutical industry as well as open collaborations with researchers leading to publication in open access journals and public/scientific presentations.

Species and numbers of animals expected to be used.

- Sheep: 755

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 produce a large volume of blood which is where antibodies are harvested from. Relatively large volumes of blood are able to be taken from sheep without any significant adverse effects. Adult sheep are used but I no longer use old sheep twoards the end of their natural lfe. During the course of the last licence, we found that thes animals had more health problems and we therefore used more sheep. The choice of adult sheep in good health allows for the maximum amount of serum production.

Typically, what will be done to an animal used in your project?

The sheep will receive several injections (up to 6 but normally less) at the same time to make up the “vaccination”. They will be injected under the skin in their neck (where the skin is loose). The total amount of liquid injected will be small (only about 0.5ml). We usually use an adjuvant, (Freunds complete) to increase the sheep immune response to the vaccine. The adjuvant contains destroyed bacteria that the sheep immuen system will react to. This increases the chances of the sheep generating antibodies to the protein of

interest. The sheep are held still for this and would only experience very mild discomfort. The sheep seem completely normal afterwards. The sheep may receive up to 3 “booster” vaccinations but would occasionally be up to 6 times. Only a very few sheep require more than 3 vaccinations (4 in the last 5 years). Animals then have blood samples taken from time to time (normally less than once a month). The first sample or two will be small to check that the sheep is producing the antibodies as expected – but then we take a bigger sample which is tolerable and does not have any adverse effect.

What are the expected impacts and/or adverse effects for the animals during your project?

The animals are well handled and familiar with the people and the technicians taking the samples are trained and very experienced. This reduces the stress for the sheep of having blood taken and I expect them to suffer no more than the pain associated with a needle prick and a little potential discomfort at being held still. During the course of the last couple of licences, the technicians lookign after the sheep have not observed any adverse effects from repeated injections or the use of adjuvants. I therefore do not expect any over the course of this licence but the sheep will be monitored by the technicans and vet.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild - all animals on procedure.

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?

Although alternative methods for using antibodies to help us work out how cells function are being developed we simply don’t have ways of mimicking the way that the immune system works at the moment. This means that we still need an animal for the production of high-quality and specific antibodies. In order to ensure that every alternative to the use of an animal is considered, each request for a new antibody will be reviewed locally by a Vet, the holder of this licence and an ethics committee. Where a requester has not shown that they have looked at all alternatives, or has not justified the work with clear benefits, the request will be rejected.

When a new project is proposed, the requester must complete a form with the background to the subject area, the purpose of the protein of interest, the expected use of the antibody and what they have done to explore alternatives. The PPL holder then conducts a search of databases to verify the target protein and to check if there are any existing antibodies

available. Should the PPL feel there are existing antibodies available that are suitable or that the proposed application for the antibody be inappropriate for a sheep polyclonal or can be achieved by a non animal alternative, then the request is rejected and returned to the requestor for further justification. This has happened 14 times over the course of the last licence. Once the PPL holder is happy, the application goes to a named vet to verify the request is ethically appropriate, in collaboration with the local AWERB. During the course of the last licence, a further 7 requests were returned after this ethical review for further justification.

For the vast majority of projects, serum is purified against the antigen and for certain types of antibody, depleted against possible non specific binders. This ensures that the final product is of the highest possible quality and utility. For all of my own projects and for all internal requesters, an antibody datasheet is required to be produced by the end user and is stored with the antibody information after each bleed from the sheep. This ensures the antibody is working for the purposes it was produced and that unnecessary further bleeds are avoided should the sheep not be producing working antibody. All antibodies produced for my research or for internal projects are made available to researchers worldwide via a weblink to our repository.
The above procedure ensures that only antibodies that are specifically required for a specific purpose or set of purposes will be produced. It also assures the quality of the product and provides future requesters of antibodies from our repository have data that validates the antibody and indicates its suitability for specific applications.

This ensures reproducibility of data.

While I have had these robust procedures in place throughout my previous licence, I acknowledge the recent ASC review of licenses for antibody production requires certain additional procedures in place. In light of this, the proposed licence will include an annual review of the operational aspects of the licence and will involve AWERB, PPL and Vet input. The annual review will consider any adverse effects or health issues from the procedures and implement refinements should they be required.

These may include adjuvant use, injection procedures and bleeding. The requester review procedure will also be reviewed in terms of project acceptances and rejections and new requirements may be implemented as a result.

Which non-animal alternatives did you consider for use in this project?

We have looked at alternative methods and will continue to do so, as well as continuing to work on developing our own. We have investigated phage display, yeast display as well as computer designed nano-bodies.

Why were they not suitable?

All of these approaches showed some potential but failed to produce robust, specific antibodies for the range of applications needed. For all the methods mentioned above, we are unable to produce antibodies that are specific in dentaured protein applications such as immunoblotting. This is primarily due to all the methods producing only fragments of antibody molecules that contain single antigen binding sites. I did note increased success when using these antibodies for immuno-precipitation procedures but equally there was an increase in contaminating proteins. Additionally, I found that quantitative measurement applications such as immuno- fluorescence were not practical with single chain antibody fragments for the majority of antigens tested. A further type of antibody that I have so far

failed to produce in vitro are for phospho-specific antigens.

I intend to continue actively researching these alternatives, and new approaches as they become available. The aim will be to develop a majority or complete replacement approach. One approach I am exploring is taking the information gained from developing a single chain antibody in an in vitro approach and then transferring this to a full antibody through recombinant cloning. While I have successfully achieved this with some reasonable results, it is extremely labour intensive and expensive and the procedure needs significant improvement to ensure reproducible results and time efficient protocols. This will be a major focus of my own research over the next 5 years.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?
I have based this on research group plans, institutional level discussions and past usage. In the current licence, we estimate 450 to 500 animals will have been used. The period of the licence covered the Covid pandemic and much mainstream research was disrupted for over a year as a result. In addition, I am working with other establishments to produce focused reagent sharing groups with focus in specific disease areas. We have completed such a resource for the SARS-CoV-2 virus and I am working with another institute to establish an influenza resource. The number proposed in this licence will allow for this work.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Each study will be assessed by the project licence holder, a vet and AWERB. Only the minimum number of animals will be allowed per antibody (usually 1). This is achieved by maximising the amount of antibody for each procedure by requiring the requester to feedback results following each bleed.

Maximum antibody titre is normally achieved by bleed 3 and this is then monitored over future bleeds. When titre falls, the procedure is normally terminated. Yields of antibody vary significantly between antigens but all antibody that is not immediately required for the requested project is stored in our repository and made available for further studies, reducing the likelihood of further animals being needed should the antibody be requested again. During the course of my last licence, there has not been an incidence of more than one sheep being needed to produce the required amount of antibody on a specific antigen.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Each study will be assessed by the project licence holder, a vet and AWERB. Only the minimum number of animals will be allowed per antibody. This has only ever been 1 during the course of my last licence. Under no circumstance would two sheep be used simaltaneously for a signle antigen. It is conceivable that an additional sheep be required if

all stocks of antibody were depleted but this request would be subject to another full review and approval would only be granted if sufficient evidence existed for the antibodies further need.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Sheep are a very common choice for antibody production, as each animal can give a large volume of blood without being harmed and they can do this on several occasions. Sheep are able to be trained and become used to being handled and sampled so that they don't find the process stressful.

During the course of my last licence, I have achieved a very high success rate in producing specific and high affinity antibodies using sheep. There have been 6 reported incidents where the antibody has not been useful for the application intended. In my experience of raising antibodies in other species, this is at least equivalent and arguably a higher success rate than in alternatives such as rodents or lagomorphs. This combined with the quantity of antibody produced means sheep are the best species for antibody production in my opinion. I have no reason to believe that any of the failed projects would have been more successful in an alternative species. The choice of adjuvant has been verified over the course of my last licence and in licences proceeding that. Combined with the observed lack of adverse effects and very good success rates, I see no reason to change this adjuvant but will monitor this constantly and monitor developments in the field.

The method of immunisation has been refined over the course of my last licence. I moved from intramuscular to subcutaneous only under veterinarian advice to minimise discomfort to the sheep. I have not observed any decline in overall success rates and will therefore continue to use this approach.

Why can't you use animals that are less sentient?

Sheep are widely considered to be a suitable species for raising antibodies. The use of sheep means maximising the amount of antibody from each procedure. This has a major advantage of ensuring reproducibility for the use of the antibody as larger amounts mean it will be used over a wider range of applications and projects. Sheep have been shown by multiple studies to be better at producing antibodies for human disease than other less sentient models such as mice or rabbits. Some antibody producers have shown sheep produce antibodies with higher specificity and broader ranger of antigen epitope recognition than other species, including other large animals.

In reality, it is the significantly lower costs and ease of re-immunisating new animals for failed projects that make less sentient animals a common choice for antibody production. The use of sheep represents an opportunity for minimising the number of procedures.

Animals may be terminally anaesthetised for final bleed.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All animals will undergo regular (monthly) health inspections by a Vet and reports made to the project licence holder. Animals will be treated accordingly for any conditions. Occasionally conditions may arise in animals that are unrelated to the procedure, but which develop in the 'normal' population, for example foot rot. Where animals show any signs of illness, animals will be treated by the Vet. In the unlikely event of an animal being on procedure for more than 6 months, the ability of the sheep to carry the right amount of oxygen in its blood will be regularly monitored.
During procedure, the sites of injection, bleeding and any adverse effects due to immunisation and adjuvant will be monitored and recorded. During the annual review with the AWERB, all data will be reviewed and refinements introduced as required.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We conform to the PREPARE guidelines. I and all involved in the care of animals during procedures will continuously monitor the literature and make refinements in line with best practice.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Primarily through regular discussion with the Vet and other staff involved in the project as well as reading all the latest scientific literature relating to advances in antibody design and production.

74. Potent monoclonal antibodies for biologics 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

Hybridoma, Monoclonal, Antibody, Therapeutic, Transgenic

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 project licence will be used to immunize animals such as mice and rats to generate antibodies that can be used as therapeutic medicines, as tools to support the development of drugs, as tools for research, and to help improve the methods currently used to discover these medicines and tools.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 aim to generate new medicines to treat diseases where there is a major unmet medical need primarily in the fields of Oncology (e.g. lung, breast, ovarian, and haematological cancers), Respiratory diseases (e.g. chronic obstructive pulmonary disease, asthma, idiopathic pulmonary fibrosis, and chronic cough), and Cardiovascular

Metabolic and Renal disease (e.g. chronic kidney disease, and diabetes), as well as microbial diseases, neuroscience and autoimmunity. The project will also help us discover tools that can accelerate clinical development of drugs as well as help us improve our methods for discovering potent antibodies.

Some of the current treatment modalities for these severe diseases include small molecule chemical drugs that have been found to have severe side effects in human patients. In addition, certain cancers develop resistance to the available therapeutic drugs. Antibodies that are naturally part of the defence mechanism of the human body are thus one of the best options to treat severe diseases with minimal side effects. The antibodies by attaching to the target cancer cell or a diseased cell type can help in the destruction of these unwanted cells from the body. Antibody drugs make up 50% of the current clinical stage drugs and are thus a major class of drugs for the treatment of a wide variety of diseases. As such antibody drugs have the potential not only to improve the outcome of disease for patients but also to cure patients of life-threatening diseases. One such example is an antibody, Panitumumab, that has been used in the treatment of colorectal cancer and has been shown to reduce the disease progression significantly. Another antibody, Imfinzi, that has been used to treat lung cancer has been shown to make significant number of patients better and keep them disease free for several years. In spite of such useful drugs, there is still an unmet need to develop newer treatments because - some of these drugs are only effective in a small cohort of patients or there are diseases where existing treatments are failing and for some diseases multiple combined treatment options are needed. Thus, we aim to generate potent, effective antibody drugs against several such diseases that are able to cure the patients effectively.

What outputs do you think you will see at the end of this project?

- Therapeutic drugs for the treatment of diseases primarily in the areas of cancer, respiratory, inflammatory, heart/lung, kidney, metabolic and neurological diseases
- High quality antibodies to understand the disease mechanism as well as to support the development of drugs.
- Such antibodies will be helpful for machine learning algorithms to learn how to make best quality antibodies. This can ultimately help to replace use of animals for drug discovery.
- Patent applications for projects successful in identifying antibody drug molecule, as well as technology developments arising from these projects.
- Where possible scientific publications and conference presentations highlighting data/learnings from the therapeutic antibody discovery programs. For example, publications directly related to the antibodies themselves, or any further understanding of the disease biology or technological advancement etc.

Who or what will benefit from these outputs, and how?

The potential benefit to patients is enormous, if the projects are successful in generating therapeutic antibodies that are useful for even a subset of patients suffering with diseases of high unmet medical need in Oncology (e.g. Lung cancer – 1.8 million new patients/year, Breast cancer - ~ 2.1 million new patients/year), Cardiovascular Metabolic and Renal diseases (which account for 20 million deaths/year), and in Respiratory diseases (e.g. Chronic Obstructive Pulmonary disease – 384 million sufferers worldwide, Asthma – 339 million sufferers worldwide).

Any new drug development can take up to 10-12 years before it can be used on human

patients. Hence, the benefits from this project are long term and may not be fully realised until the completion of the project. At the beginning of each study, we identify a target drug profile e.g. required potency, specificity which tells us about the criteria of a successful drug for that particular disease. Once a panel of antibodies is identified fitting this criteria, we further ensure thorough analysis of these molecules in terms of their physical properties, suitability for manufacturing and a computer simulation based analysis on their dosage in humans. These studies help us identify the right antibody molecule that is likely to succeed through the clinical development. In this way, we ensure the successful onward development of the antibodies.

As mentioned, we also aim to generate tool antibodies that can help support the development of therapeutic drug antibodies. These tool antibodies are required to detect the therapeutic drug antibody or to detect the anti-drug antibodies in human patient samples in clinical studies. There are human drug antibodies which do not show equivalent functionality in animal models. Thus it becomes difficult to study such drug molecules in pre-clinical studies. In such cases, parallel reagent antibodies are generated having similar properties like the drug molecule but showing functionality in the animal models. This helps in the progression of drug antibodies through the pre-clinical study models. Such tool or parallel reagents are routinely required at relatively short notice. Hence, the benefits coming out from such studies are likely to be realised within the lifetime of the license.

Once an antibody is patented after discovery (~18 months) the data can be published or presented. This is likely to happen within the lifetime of the license. This can benefit a wider scientific community in terms of dissemination of data, and information relating the antibodies and other reagents generated in the form of publications, patent applications, conference presentations/proceedings etc. Where possible antibodies and other reagents generated for each project may be used with external academic collaborators to help further understand target biology and/or develop novel technologies to improve scientific methodologies e.g. we are using antibodies and their hybridoma cell lines to investigate and develop technologies to improve antibody isolation using microfluidic techniques.

How will you look to maximise the outputs of this work?

We will look to publish manuscripts and patents, as well as present our work at conferences regarding the discovery of new drugs, tool reagents and developments of new methods as well as novel insights into the biology of diseases. We will also consider publishing unsuccessful approaches and non- significant data via open access platforms such as F1000Reseach.

Species and numbers of animals expected to be used

- Mice: 2300
- 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.

Small animals such as mice and rats are the animal species of choice for antibody isolation because they are easily handled and easy to care for. The immune response in these animals is well understood, is generally robust and reproducible.

Immune systems of mice and rats are similar to human immune system that help generate large and diverse types of potent antibodies. Furthermore, in vitro (in laboratory) isolation of antibody genes from the cells of these animals to help generate antibodies on large scale in laboratories is well understood. Adult animals (typically 6-8 weeks old, not exceeding 6 months of age) of either sex (male / female) are typically used for generating antibodies, as the immune response is immature at an early age and weakens in older age. Certain human disease targets can have equivalent (homologous), functional counterparts in mice. Hence, it is difficult to generate an immune response to such targets in mice. In those cases, we would use rats to generate antibodies.

Typically, what will be done to an animal used in your project?

Typically, at the start of a protocol, animals of appropriate age will be labelled (to identify individual animals), weighed, and blood sample will be collected as a pre-immune sample. Depending upon the protocol, animals will be injected with an antigen (substances such as proteins/RNA/DNA/cells against which antibodies are generated) via suitable routes such as subcutaneous (under the skin), intra peritoneal (in the stomach cavity) etc., multiple times over the duration of the protocol e.g. 28 days.

Animals will be regularly monitored for characteristics such as weight, behaviour, appearance in between the injections.

During the in vivo (performed in or on living animals) experiment, blood sample will be taken to test the generation of an antigen specific antibody response. There may be a small number of studies where we would be interested in imaging the animals in a non-invasive way to check the expression of a target antigen upon genetic immunization (using RNA / DNA). This will enable us to develop and optimise this immunization strategy, which has the promise to improve immune response to challenging drug targets (complex targets on the cell surface), which are a major class of therapeutic target (~30% of all marketed drugs).

At the end of the protocol, animals will be humanely killed and organs such as spleen, lymph nodes and bone marrow will be harvested. The B cells (cells secreting antibodies) from the harvested organs will be used to discover potent antibodies against the target antigen using established laboratory methods.

What are the expected impacts and/or adverse effects for the animals during your project?

100% of the animals are likely to experience mild levels of severity such as transient discomfort/irritation at the site of injection, transient weight loss. This is because they will undergo repeated administration of substances and sampling using standard routes. Unless otherwise specified, the administration of substances and withdrawal of body fluids will be undertaken using a combination of volumes, routes and frequencies that of themselves will result in no more than transient discomfort and no lasting harm. Animals are not expected to show any deviation from normal health and behaviour. The mentioned adverse effects are expected to last only for a short duration and can be managed by, for example, administration of food enrichments. Based on our experience on previous licenses, few incidences of transient anaphylactoid responses may be observed after the

last injection that do not cause long lasting harms. The symptoms might include one or more of the following: swollen snout and/or paws, piloerection (erection of hair), hunched posture, inactivity but responsive.

These symptoms typically manifest within 2 hours post-immunization. Such animals will be monitored closely. These symptoms usually resolve without any intervention within 2 hours of onset. If no sign of improvement is observed over the observation period the animal will be humanely killed. In case of a severe anaphylactoid reaction, such as seizures typically occurring within 15 minutes of dosing, the animal will be killed immediately.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice
Mild Severity - 100%
Moderate severity - 0% Rats
Mild severity - 100% Moderate severity - 0%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The use of animals in this project is required to generate effective antibody medicines and antibody tools which could help treat different types of diseases with high unmet medical need (e.g. cancer, diabetes, asthma). Immune systems of mice and rats have natural capabilities to generate high affinity (a natural property to attach to the target specifically and tightly) antibodies to the target antigen (a substance to which the antibody binds) with the help of processes such as affinity maturation where cells secreting high affinity antibodies are gradually selected over the ones secreting low affinity or non-specific antibodies. Since the antibodies are generated inside a living animal they are naturally selected by the immune system for beneficial characteristics such as specificity to the antigen and absence of any unnatural structural changes. With the use of genetically modified mice expressing human antibody genes, high affinity human antibodies useful for therapeutic purposes can be easily derived.

While there are in vitro (in laboratory) technologies for antibody generation available, these methods are not suitable for all disease targets. For example, it is extremely challenging to generate medicines to disease targets which are found on the surface of cells using in vitro technologies. Antibodies to simple soluble protein targets (not attached to cells) can be generated using in vitro technologies.

However, to generate a potent drug out of it, each antibody derived in vitro will need to

undergo further in vitro manipulation which is often very long, require great efforts, and typically performed on one antibody at a time. This could result in significant delays in bringing therapeutic drugs to patients in the clinic. Additionally, the process of improvement can have other side effects on the drug making it difficult to manufacture and the potency of the drug cannot be guaranteed.

A review of the most appropriate approach for generating our medicines will be carried out at the beginning of each programme. We will actively search for, sought access to, and interrogate third-party suppliers and libraries to confirm, if the antibody that we desire is already available. We will also apply the learnings from successful projects carried out on this licence to help build our knowledge of how to make in vitro antibody generation technologies more reliable in future.

Furthermore, we have introduced new technologies to allow us to perform deep searching of B-cells generating potent antibodies directly from immunized mice. This new technology has the power to generate very large antibody data sets which will be used to help train machine learning (use of computers to analyse data and identify patterns) algorithms. The aim of this initiative is to determine if we can train machine learning algorithms to design high affinity antibodies in silico (using computer simulation) to ultimately replace both in vivo and in vitro antibody discovery technologies. The datasets generated from in vivo studies are especially valuable, as these antibodies have been generated naturally to the target antigen, and then affinity matured by natural processes. Currently, there is no in vitro system that can be used to model the complex antibody generation process that the in vivo antibody generation and affinity maturation provides.

There has been significant scientific discussion about the justifiable use of animals for therapeutic biologic drug discovery after EURL ECVAM recommendation on non-animal derived antibodies (European Commission, Joint Research Centre, Barroso, J et al 2020 https://data.europa.eu/doi/10.2760/80554). However, it has been clarified separately by few of the original authors on this report, that these recommendations for non-animal derived antibodies were made excluding therapeutic antibody discovery and development (Bradbury et al, MAbs 2021). The authors also further opine that currently there are significant challenges in widespread adoption of in vitro platforms that could compete with / replace the in vivo biologic discovery process. Until such challenges are overcome, it is important to keep both options in the future for therapeutic antibody discovery (Bradbury et al, MAbs 2021). In addition, European Animal Research Association (EARA) and the European Federation of Pharmaceutical Industries and Associations (EFPIA) along with others have also called these recommendations as premature highlighting that – animal derived antibodies are critical tools from basic research to development of life saving drugs, non-animal derived antibodies cannot recapitulate all the useful properties of animal derived antibodies and as evident recently from COVID-19 research, animal derived antibodies play key role in development and large scale production of therapeutics (https://www.eara.eu/post/eara-efpia-response-to-antibody- recommendation). Thus, it is significantly valuable to generate potent antibody drugs by immunizing animals which could help treat millions of patients worldwide.

We have also considered the recommendations made in the report by Lord Sharpe: 'Review of antibody licences: letter from Lord Sharpe'. We have made attempts to thoroughly review our 3R strategy accordingly.

Which non-animal alternatives did you consider for use in this project?

We have access to in vitro antibody discovery technologies such as phage display, a

technology where antibodies are displayed on the surface of a virus particle. Before starting a new immunization campaign a full scientific review of the target antigen and antibody requirements will be carried out which will enable us to choose the most appropriate antibody generation platform (i.e. in vivo vs in vitro) for each target. There will be target types and specific requirements where phage display may be the only route to generate antibodies e.g. where there is a high degree of similarity between human and mouse antigens, a high affinity antibody is not required and/or where a very specific region of a protein is required to be targeted where in vitro display technologies would have an advantage over an in vivo approach.

Both in vivo and in vitro derived antibodies / datasets will help develop our machine learning and in silico antibody discovery platforms, which has a potential to replace both the discovery platforms in the future.

We are also aware of the NC3Rs website and database which we will access regularly to receive latest updates on non-animal alternatives.

We would also plan to engage AWERB on selecting non-animal derivatives, by presenting our findings/ progress with the committee regularly. In addition, we can discuss some of our up coming projects and discuss our justification for the use of animals. We can also share any novel non-animal technologies with AWERB forum to receive feedback on non-animal derivatives in our projects.

Why were they not suitable?

In vitro antibody discovery technology is likely to generate artificial antibodies which may not exist naturally in the human body. This can give rise to undesirable properties in those drug molecules which are identified later on during their development process. Improving upon these unwanted properties in a drug can delay the development of medicines for unmet needs. Currently, there is no in vitro system that can be used to model the complex antibody generation process that the in vivo antibody generation and affinity maturation provides. It is known that isolating functional antibodies to targets that are expressed on cell surface is challenging for in vitro technologies. Where large numbers of high affinity antibodies are required as drugs or as tools, the restricted use of in vitro antibody generation methods would result in significant delays in the progression of drug molecules into clinical trials in humans by years which, for the major diseases which we aim to tackle such as cancer, diabetes, asthma, etc., could potentially result in many more patients dying from such diseases than 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?

The number of animals used per project will be based on the aims for the particular study, for example, whether the requirement is a drug or a tool antibody, mode of action required (e.g. binder to target, neutralizing a target etc.), our previous experience with a similar

project, available knowledge and the likelihood of success. Therefore, the number of animals used per project will vary with the target type and aim of the project. Based on the previous license experience, as well as scientific research and consultation with experts within the company, 3 groups each consisting of up to 6 animals is generally sufficient to obtain the required results for experiments involving therapeutic antibody discovery.

However, for certain targets, based on prior experience, groups sizes as low as 3 animals per group can be used to generate a successful outcome for the given project. We will also explore the possibilities of combining pilot studies for new studies along with our studies to refine our procedures resulting in usage of fewer number of animals. We have set a conservative figure of 2300 mice over the 5-year license period. This is with the assumption of doing 100 drug discovery projects and 25 tool discovery projects over the span of 5 years.

Rats are occasionally used in the generation of tool antibody reagents, that help in the development of human drug molecules. For rat studies, we use 3 rats per group, up to three groups per study. For our previous licence, no projects have required rat immunizations to date. However, we would like to retain rats as an option should the need arise and estimate 50 rats would be used over the course of this licence. As a demand led PPL, it is difficult to predict total animal usage.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We prepare a statistical health check report for our studies that includes details about the study design such as study groups, planned data analyses, assumption of the analyses, justification of animal numbers and how we control the bias and variation.

In vivo protocols are designed to use minimum number of animals to achieve study objectives. Our immunization protocol allows us to generate strong antibody responses over the shortest immunization time possible, helping us in efficient use of number of animals. We used our experience from previous experiments conducted on our past licences over the past 15 years, as well as literature searches and consulted with experts within the company to guide the design of our studies where appropriate.

I am familiar with the online tools available for help with the experimental study design such as NC3R’s Experimental Design Assistant. I have also read literature on designing experiments with appropriate sample size on FRAME (Fund for Replacement of Animals in Medical Experiments) website: https://frame.org.uk/resources/experimental-design/. The availability of expertise and the literature/online resources have helped guide the experimental design.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will use our experience from previous experiments, literature searches and consultation with experts within the company to guide the design of our studies where appropriate. When we are investigating a new method of antibody discovery, where possible we will combine these activities with real drug discovery projects in order to minimise the number of mice used, whilst still giving us a comparator groups of standard best practice. Where appropriate, small pilot studies will be conducted to ensure that the study objectives can be met. We have developed a new method where we can screen antibody secreting B-cells directly for antigen binding. We are also working on cutting edge

platforms where we aim to screen B cells directly for functional antibodies. Thus, employing multiple antibody discovery platforms will help us maximise the output from the immunized animals and eventually reduce 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 and rats are small, easily handled species with highly characterised immune systems and well- defined biology. Mice and rats are short lived, have rapid generation times, and are easier to look after than other larger animals. We plan to use our proprietary transgenic mouse strains and other commercial strains (e.g. Ablexis) to generate fully human antibodies. We also plan to use wildtype mouse strains such as CD-1 , NZB as well as wildtype rat strains. For studies requiring generation of fully human therapeutic antibodies we use transgenic mice whereas for tool / reagent generation studies, we use wildtype strains. Hybridoma technology that generates immortal antibody secreting cells in laboratories using the cells from animals is routinely used for antibody discovery. The animals are kept in high quality pathogen free facilities by highly trained animal care staff to ensure that the animal welfare is paramount. Animals are given environmental enrichments and will whenever possible be group housed.

The antibody response in mice is well understood. The injection routes used in this project have all been shown to cause no adverse effects (i.e. Mild severity) whilst inducing effective antibody responses in the majority cases.

Rats tend to be used when the target antigen is mouse or similar to mouse. Because it is difficult to generate an immune response to a 'self like' substances in animal bodies.

Our immunization protocol allows us to generate high quality, high affinity antibodies over the shortest period while maintaining a high level of animal welfare. Our immunization protocol is relatively short in duration that helps the antibody secreting B-cells to be predominantly located in specific small organs called lymph nodes.

Longer immunization protocols would result in B-cells migrating to other organs making it difficult to isolate such desirable B cells. Shorter immunization protocols are described in the literature, however, these shorter protocols involve far more immunizations per time point and are likely to have a significant impact on animal welfare, therefore, we do not use these.

Why can’t you use animals that are less sentient?

Mouse / rat immune system is well studied and there is a significant knowledge available in the literature about various routes, procedures, and outcomes with respect to immunizations. Based on the scientific data and our previous experience, we intend to use

adult animals of either sex, typically 6-8 weeks old, not exceeding 6 months of age. The animals of adult stage have an active, fully functional, mature immune system as compared to being either immature at an early stage or weak as the animals get older. Hence, we prefer to use animals of adult stages.

To generate antibodies, animals are injected multiple times with the antigen. This process takes advantage of the natural mechanism of the immune system where repeated exposure to the antigen allows for generation of potent, high affinity antibodies. Thus, it is required to use animals of adult stages which can be efficiently immunized over a period of few weeks.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have been working to refine our immunization practices, as mentioned in the previous PPL licence. Using our original immunization protocol where mice were immunized sub-cutaneously while awake, we found antibody responses to our targets were variable within a group of mice despite all receiving the same dose of target. Furthermore, we saw an incidence of severe immune reaction (anaphylactoid) which, when it occurred, often resulted in the mice having to be culled as they did not recover within agreed timeframes for this mild severity licence, or in extreme cases the mice died shortly after the injection. This incidence rate was calculated to be around 2% year on year.

Our revised procedure now involves administering the sub-cutaneous injection while the animals are under general anaesthetic (a substance that induces insensitivity to pain). This is much easier for the in vivo staff to administer, and placement of the needle and the injected material is far more accurate.

The result of this change has meant that we now have a far more even antibody response to our targets within a group of mice. This has enabled us to reduce mouse numbers per group on many projects. Furthermore, the incidence of severe reactions to immunization has decreased significantly, whereby very few mice have had to be culled or have died during the procedure. In addition, whenever required we aim to ameliorate animal harms by providing nutritious food supplements, regulated temperature and analgesics (substances that relieve pain).

We will investigate further, to see whether we can ultimately reduce the number of immunizations given to each mouse per project. We will also investigate whether giving the final boost sub-cutaneously can help to reduce the incidence of any anaphylactoid like effects occasionally observed at the final dose with intra-peritoneal immunizations.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will adhere to the published best practice guidelines from LASA to conduct our experiments. We will also follow ARRIVE guidelines to ensure in vivo experiments are planned and carried out in the most robust and reliable manner. We are also aware of the PREPARE guidelines, which will be used to increase the reproducibility and quality of our research.

Our study on antibody generation requires us to use small adult rodents which have mature immune system and thus are able to provide us with potent antibody drugs. Currently, there is no animal model available that is less sentient than rodents and could

provide potent antibody drugs for the life threatening diseases that we aim to counter.

We are also committed to the ethical use and welfare of animals. Learnings from the previous PPL license will help us conduct our experiments in the most refined way.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will keep up to date about the advances in the 3Rs by regularly accessing the online resources such as NC3Rs and FRAME. Before starting a new animal immunization campaign, we will carefully review the pros and cons of such an exercise as well as thoroughly analyse whether an alternative in vitro methodology can be used as a replacement. This will help us achieve our 3R aims. We also aim to explore new technologies for antibody discovery and seek to reduce the animal usage as well as refine our techniques.

75. Production and Maintenance of GA rodents

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Production, Maintenance, Genetically Altered Mice, Cryopreservation

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant
Rats	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To produce unique strains of genetically altered (GA) Laboratory mice as required by various research programmes within the establishment.

To remove potentially harmful diseases and organisms through a process called rederivation thus improving their health status.

To freeze tissues from genetically altered mice for future use.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

This licence improves efficiency by centralising a service and allows a core of highly skilled individuals to provide a service allowing production of new lines/strains of mice without the need for continuous training of new individuals. By utilising a small team of highly trained

technicians the procedures involved will be carried out to the highest possible standards.

It will allow unique genetically altered strains of mice to be produced and go on to be used in applied human medicine research programmes.

It will allow strains to be cryopreserved (deeply frozen) for future use or transportation reducing the number of animals or the need to transport live animals.

What outputs do you think you will see at the end of this project?

As a service licence the outputs of this project will be:

- new genetically altered mouse strains created for use in further studies
- healthy, disease-free animals to be used for further study
- genetic material (rodent embryos or sperm) stored for either transport to other institutions or held until such time they are required

Who or what will benefit from these outputs, and how?

These outputs will benefit the wider research community by providing:

- a means to generate genetically altered animals relevant to their project that they do not have the expertise to do themselves
- a means to transport animals from institution to institution without the need to use live animals
- a means to establish a colony of genetically altered animals managed by experienced personnel whilst applying for their own project licence or, under specific conditions, retain a colony short term while the researcher is waiting for a licence renewal
- advice and expertise in the relevant colony management and surgical techniques underlined in this licence

How will you look to maximise the outputs of this work?

Although there will not be any scientific data produced by this service licence, we will present any relevant developments or refinements considered beneficial to the wider animal technology industry, either through publications or meetings as well as regular communications with other institutes carrying out similar work.

Where possible, any excess animals produced will be offered to local researchers for tissues or training.

Species and numbers of animals expected to be used

- Mice: 6100

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Breeding and the genetic alterations affect all systems and as such live animals are still required. Mice are the most appropriate species for this as:

- the entire mouse genome has been mapped.
- they are relatively easy to manipulate on a genetic level.
- the can produce in a short space of time, a large number of genetically identical animals.
- The majority of animals used under this licence will be adults, used to generate embryos. All other life stages, from egg to adult will be used to create or maintain genetically altered colonies. There is no current plan to generate genetically altered rats under this PPL but genetically altered rat lines may be bred and maintained under this PPL for short periods prior to transfer to another suitable licence.

Typically, what will be done to an animal used in your project?

Animals under this project will be subjected to the following:

- Hormone injections
- Vasectomy surgery
- Embryo transfer surgery

Additionally, embryos (eggs) from Mice will be used to create new genetically altered Mice by either adding DNA or taking it away. To create a new genetically altered type of mouse scientists will either add or take away a specific part of a gene or specific DNA by inserting it into a growing mouse egg either by injecting directly into the egg or using a machine to temporarily dissolve the eggs protective layer to allow the DNA to enter. This egg will then be transferred to a new “mother” mouse and allowed to grow. When the mouse is born it is hoped to have taken the modification and once old enough can be bred so that it passes on the same modification to its offspring allowing researchers to study it and the effects of the missing or added DNA.

Regarding the genetic alterations themselves the vast majority of animals are normal and will show no changes in behaviour, have no health implications and show no noticeable detrimental effects.

Much like in human in-vitro fertilisation (IVF), the mice are given a series of small hormone injections (normally this involves 2 separate small injections of 0.1ml) designed to supercharge the mouse ovary into producing lots of eggs and ensure they are in the right place to be collected. The mice will suffer only very mild discomfort and return to normal quickly. This will be carried out by experienced staff trained in the procedure. 24hrs after the second injection the mice are humanely killed and any eggs (embryos) carefully collected. These eggs can then be frozen and stored for future use or manipulated to create new genetically altered rodents.

Vasectomy surgery (preventing a male from fathering offspring by surgically cutting relevant tubes) is carried out under general anaesthesia using strict sterile techniques. It is a short surgical procedure taking approximately 30-40 minutes. The animals are given medicine to relieve any pain throughout and monitored closely for the duration of the surgery and should return to normal behaviour quickly. They can be mated to produce “pseudo-pregnant” female mice (this convinces the female mice that they are pregnant, and they begin to go through the process as if they were allowing any implanted eggs to hopefully develop normally.) Post-surgical infection is extremely rare but any mouse

showing any post-surgical complications or infection will be checked by a vet and either treated or humanely killed. Vasectomised male mice will be housed on their own but given a female companion animal when not required to generate pseudopregnant females.

Embryo transfer surgery (transferring a modified egg into a new mouse) is also carried out under general anaesthesia using strict sterile techniques by highly trained staff. It is also a short surgical procedure taking 30-40 minutes. Following any modifications to the eggs as described above, the fertilised embryos (originally from donor female mice) are transplanted through a small cut into the oviducts of pseudo-pregnant female mice (recipients). The animals are given medicine to relieve any pain and monitored very closely throughout the surgery and expected to return to normal behaviour quickly. Post-surgical infection is extremely rare but any mouse showing post-surgical complications or infection will be checked by a vet and either treated or humanely killed. If successful, the mice are allowed to give birth and kept with the offspring until they are old enough to fend for themselves.

Unless required for health screening (to check if there are any unwanted diseases or pathogens) the recipient mothers are then humanely killed.

All animals will be housed in appropriate caging and environment suitable for the species with free access to food and water. Appropriate enrichment for the species will be provided.

Animals welfare will be checked at least once daily by competent technicians.

What are the expected impacts and/or adverse effects for the animals during your project?

Surgical procedures will have the largest impact to the animals.

Both Vasectomy and Embryo Transfer surgery are short procedures lasting no longer than 30-40 minutes. Animals will be under anaesthesia the whole duration of the procedure and given pain relief medicine before and after. They are expected to fully recover within a few hours from the anaesthesia and a few days from the invasive surgery.

Injections are short and involve a very small amount of drug or hormone.

Any animals showing signs of distress, ill-health, abnormal behaviour or significant weight loss 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)?

Injection of hormones (Mice): 100% Mild Vasectomy surgery (Mice): 100% Moderate
Embryo Transfer Surgery (Mice): 100% Moderate
Breeding and Maintenance of GA Mice: 10% mild and 90% sub-threshold

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?

Breeding and the genetic alterations affect all systems and as such live animals are required.

Mice are the most appropriate species for this as:

- the entire mouse genome has been mapped.
- they are relatively easy to manipulate on a genetic level.
- the can produce in a short space of time, a large number of genetically identical animals.
- they are the main species used in medical and basic research.
- nevertheless, we are constantly reviewing procedures and current literature to look for alternatives.

Which non-animal alternatives did you consider for use in this project?

The area most likely for non-animal alternatives within the scope and timeframe of this licence will be the replacement of the need for vasectomised males, either with chemical or physical alternatives.

Currently an available alternative is to use naturally sterile males (thus not requiring any surgical procedures) but this is a refinement not a replacement.

Why were they not suitable?

There is currently not enough evidence to suggest they are a suitable alternative but it is something we may investigate ourselves within the timeline of this licence.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Approximately 6100 mice over 5 years. However this is based on the previous experience and assumes that this production licence will be used regularly throughout its lifespan.

What steps did you take during the experimental design phase to reduce the number

of animals being used in this project?

We will use well established protocols to maintain good consistency and will continually review animal numbers (along with the protocols) to reduce any wastage.

Efficient colony management using current "best practice" guidelines will also be utilised to further reduce the amount of animals used in this project.

Careful scrutiny of service requests and associated justification will be made involving local ethical review process to ensure animals are not used unnecessarily.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Minimum numbers will be used by ensuring that any mouse colonies are kept at the lowest possible size to keep them going for as long as required. Careful management of the colonies will ensure no excess animals are produced.

Where possible the most up to date techniques will be employed to ensure that the maximum number of embryos are gained from the smallest number of mice.

Protocols are constantly reviewed and compared with industry best practice to allow for maximum efficiency.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use adult mice for all surgical techniques outlined in this PPL. They will be carried out under general anaesthesia and use a comprehensive regime of pre and post operative care by dedicated animal technicians. The techniques involved are well established across the industry and the animals are expected to make a swift and full recovery.

Hormone injections will only be carried out in adult mice and will involve 2 small injections not causing lasting harm to the animals again, using well established protocols.

Why can’t you use animals that are less sentient?

For the majority of this work, mammalian embryos are required as well as a comparable biological system to humans. Mice have long been established as the best species for this as well as having a genome that has been comprehensively mapped. Currently most of the basic research globally uses the mouse.

How will you refine the procedures you're using to minimise the welfare costs

(harms) for the animals?

Procedures carried out under this licence will be performed by experienced technicians working closely with the veterinary staff.

For Surgical procedures detailed post-surgery monitoring sheets are in place together with additional checking (if required) to ensure the best possible care for animals recovering from surgery. Only healthy animals of an appropriate age will be considered for surgery.

It is also planned to continue to develop and increase our use of non-surgical embryo transfer techniques and investigate alternatives to using vasectomised male mice throughout the lifespan of the licence.

All genetically altered lines are closely monitored and each have a detailed GA passport that outlines the nature of the alteration, historical data regarding breeding of the line and any potential welfare issues allowing swift action to be taken should any unexpected patterns develop.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

For regulated procedures carried out under this licence RSPCA/PREPARE guidelines will be used (where applicable) in the planning of studies with each plan of work reviewed by senior animal technicians and the vets prior to starting.

Requests to generate new GA mouse lines or hold existing GA lines will be considered by our local ethical review body in addition to the above.

We will consult other resources including guidance and publications from the Home Office, 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 together with online webinars. We receive information regularly via meetings and presentations from staff in the wider research or animal technology community.

Additionally, we have regular meetings with other universities to discuss current or potential refinements and a dedicated team of technicians involved with the work who will actively research new developments and training.

76. Adaptation in the musculoskeletal system

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Muscle adaptation, ageing, transcription, hypertrophy, wasting

Animal types	Life stages
Mice	adult, aged, juvenile
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?

Muscle is a plastic tissue that adapts in response to changes in activity or loading, and is subject to deterioration with ageing that significantly decreases quality of life and independence for many persons. We use models in rat and mouse to uncover the mechanisms of such adaptation and decline to understand and potentially to design safe interventions to improve musculoskeletal health.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Changes in human musculoskeletal health are known to involve the muscular and nervous systems. Small mammals such as rats appear to show very similar transcriptional responses to exercise as humans and suffer decline of motor function as a result of interdependent failures in the muscular and nervous systems. We want to make longitudinal studies of these interactions over the lifespan. This cannot be achieved in organ or tissue culture systems because culture systems do yet replicate such interactions, as they happen over weeks, months and years in the adult mature musculoskeletal system. Therefore they can be undertaken in rodents, whose natural lifespan is a few years. We believe that we can follow the specific interactions between muscle activity and changes in cell function in rats and mice, and then look for those same mechanisms in human so that we can potentially intervene in the future to prescribe exercise more intelligently, to possiby enhance the benefit of exercise, and to reduce the short-term detriment of inactivity.

What outputs do you think you will see at the end of this project?

Time courses of changes in how muscle genes are turned on or off, and how muscle proteins are generated and degraded with increased and decreased activity. Identification of the genes that drive muscle growth and wasting.

A definitive answer to the question of whether progressive motor neurone (nerve) dysfunction is the cause of muscular decline with old age and is a preferred target to reduce decline in the musculoskeletal system.

Tests of computer generated predictions of how electrical stimulation causes nerve activation, especially when that stimulation is applied to the surface of the body.

Evidence to show the effect of reduced Vitamin D receptor expression on changes in muscle in response to strength or endurance training. This will show how important Vitamin D, normally produced in the skin on exposure to sunlight, is to musculoskeleatl health. And whether nutritional supplementation of Vitamin D should be recommended, especially in human populations that receive relative low exposure to sunlight.

An analysis of the effect of overexpression of a variant of SIRT6 in mice. This enzyme is important in DNA repair, how cells use sugars, and inflammation. Humans with the variant that we will test have a high incidence of longevity, so if we can understand how this variant changes cellular and whole body function in mice, we may be able to harness some of its beneficial effects for healthier ageing in humans.

Continued publication of our work in influential journals such as FASEB Journal (Federation of American Societies for Experimental Biology), Journal of Physiology, American Journal of Physiology, Scientific Reports

Who or what will benefit from these outputs, and how?

Other members of the international scientific community interested in musculoskeletal biology, by evidence gathered through our expertise and resource in applying programmed activity in rodent models.

The ageing human population and their carers who need evidence-based strategies to maintain musculoskeletal health into old age.

Those interested in the application of functional electrical stimulation for rehabilitation and restoration of function, by critical analysis of the practical utility of numerical simulations of nerve activation beneath the skin to select electrode positions and patterning of the activating pulses.

How will you look to maximise the outputs of this work?

By regular presentations at national and international scientific conferences and symposia that address practical application of electrical stimulation for rehabilitation .

By purposeful public engagement planned as part of our motor unit ageing study

By use of social media account to highlight new findings to a more general audience of sport science and rehabilitation professionals

Species and numbers of animals expected to be used

- Mice: 120
- Rats: 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.

We have considerable experience of interventions to increase or decrease muscle activity in rats and mice. We have shown that the transcriptomic responses to these interventions have considerable commonality between human, rat and mouse, but important and fascinating differences that seem to be related to the actual response in terms of muscle growth. We can use such differential gene expression to focus on the genes and pathways that are most directly related to the processes of muscle growth and wasting. Because our model allows us to follow muscle adaptation over a period of weeks, we can follow not only the initial response (as is sometimes possible in cell culture) but also the adaptation that mature muscle makes to accommodate a new pattern of activity. Such training responses are of course essential for athletic performance and for the use of exercise in rehabilitation or to improve health, but they take place over a longer time period than can be

investigated in cell or tissue culture. There is increasing evidence that work in rats might be more easily translated to inform human physiology and pathology than work in mice, especially in research into the process of ageing.

We generally work in the adult phase of rodent life, once musculoskeletal maturity has been reached, and into old age. For mice this means from about 2 months of age to 18 months. For rats, typically from 2 months of age to 36 months. We are interested in the changes in muscle phenotype that are possible once the cells in the muscles are no longer subject to the influences of rapid growth in the early stages of life, and how responsiveness to exercise changes with ageing.

Typically, what will be done to an animal used in your project?

Typically, mice or rats will receive a sterile implant and a system of subcutaneous stimulating electrodes to increase, or cannulae delivering a nerve blocker to decrease, the activity of a group of muscles in one hind limb over a period typically of about 4 weeks. The implants are as small as possible and do not inhibit movement or normal behaviours such as running and climbing. The activity delivered by the implant can be programmed and adjusted so that exercise is delivered according to a repetitive daily or weekly schedule without the need for further human intervention.

Some animals will undergo a period of changed activity and then have muscles harvested after humane killing for analysis. Others will undergo repeated measures of muscle size by non invasive imaging and/or motor unit number under anaesthesia up to once a month and in some cases, into old age to investigate progressive changes in the neuromuscular system.

Some mice, including cohorts of transgenic mice, will have repeated standard behavioural tests of motor coordination and grip strength, and measurement of cardiac function by echocardiography (ultrasound).

Some rats will additionally have their diet changed to induce obesity, to investigate whether overweight changes the decline in musculoskeletal function into old age.

Some rats will have a procedure under anaesthesia to change the expression of genes within their hind limb muscles by the process of electroporation of the muscle tissue to allow the ingress of modified genetic material to change the expression of proteins of interest, such as the vitamin D receptor, or components of the pathways anticipated to be involved in ageing mechanisms.

What are the expected impacts and/or adverse effects for the animals during your project?

The impact of surgical procedures will be managed with aseptic technique and careful anaesthesia and analgesia. Recovery from the surgical procedures is usually complete within one or two weeks. We have many years of progressive development of the

techniques that we will apply and have used these techniques with a very low rate of adverse effects such as wound breakdown.

For the ageing rats that we will use, there will be the expected loss of condition in the third year of life, but we have chosen an F1 cross between Brown Norway and F344 rats that suffers fewer age related complications than other strains and is recommended by NIH in the USA and supplied to their funded researchers for ageing studies. We have begun a successful breeding program to provide stock of this strain.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mouse: 70% mild 30% moderate

Rat : 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?

No numerical simulation or tissue culture system can reproduce the changes associated with ageing or extended exercise training to induce muscle growth or disuse to induce wasting. We keep abreast of developments to improve the maturity of nerve muscle constructs in culture, but at present there is general agreement that they can only represent developing or immature muscle. Further development is required to incorporate nerve and connective tissue elements into muscle cell culture. In a recent survey we made of experiments to induce changes in cultured muscle tissue by programmed activation we found that the literature is contradictory and confused. In many cases stimulation is applied without confirmation of any resultant contractile activity. For now, we need at least some capable teams such as ours to continue to work on mature models in adult rodents and to publish our work with close attention to the methodological details so that future attempts to replicate the changes noted in mature muscle and advance understanding can be intelligently planned and monitored.

Which non-animal alternatives did you consider for use in this project?

Cell and tissue culture as discussed above. Numerical simulation of neural activation.

Why were they not suitable?

Numerical simulation of neural activation, and musculoskeletal responses to such activation is a key part of our ongoing project and may in future become a genuine alternative but for now needs further data collection, critical testing and refinement. This is a stated aim of our work.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have an experiment planned that will use approximately 80 rats in groups of 6 to follow changes in the motor unit number and muscle growth and wasting into old age in male and female. The calculation of the number required is based on previous work to follow the reduction in motor unit number in old age and the anticipated variability in our measures of motorneurone number by microscopical analysis of the spinal cord.

We have a PhD project tocontinue transcriptomic analysis of the exercise response in rats that we expect to use 40 rats, based on our published transcriptomic work since 2022. In this work we have found that groups of 4 rats at each of four timepoints (2,10, 20 and 30 days) gives a good overview of the timecourse of progressive change in the acute response to exercise over a period of training. We will be able to test two new patterns of training over this same timecourse using another 40 rats with 8 acting as sham controls.

We wish to determine the phenotype a new transgenic strain of mice in a colony of about 100. We will house three strains of mice, wild type, SIRT6 variant transgenenic and wild type control transgenic.

From each strain we will establish separate groups of 5 males and of 5 females, to be assessed by behavioural and cardiac functional testing and then harvest of organs for biochemical analysis at 3 life stages (3 strains x 2 groups of 5 x three harvest timepoints = 90 mice). We will use another 20 in our project to continue transcriptomic analysis of the exercise response in mice. This is enough for a single additional timecourse experiment in which we will add the use of deuterium oxide to follow protein synthesis and degradation to try to understand why resistance training produces less muscle growth in mice than it does in rats.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The planned experiments are a continuation of a long and productive project to understand exercise and ageing responses in rats and mice, so we can base our calculations of likely effect sizes on our previous work and, where we are introducing new techniques such as echocardiography in mice, on published data. We maintain access to the powerful statistical and bioinformatic tools available to analyse whole genome transcriptional data by subscribing to Partek, and have found in work recently published in The FASEB Journal that we can follow the timecourse of transcriptional change with surprising repeatability and statistical power with rather low group sizes such as 4 or 5 at each timepoint . This is at least partly because we use interventions such as neuromuscular stimulation or neural silencing in one hind limb, and have shown that while the contralateral limb is not unaffected, it is a very useful control so that we can investigate fold changes between test and control limbs in individual subjects.

We have invested some considerable time to assess the usefulness of the Experimental Design Assistant tool. We feel it needs further development to provide additional refinement of our experimental design, especially in timecourse experiments such as we perform, in which repeated measurements are just as valuable as strict blocking and rigid timing of measurement.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The breeding for the F344xBrown Norway is in house so we can control the number of F1 progeny that we produce for this specific project and do not need any excess.

Data from experiments on neural activation including interferential stimulation will be communicated via our weekly meetings with colleagues with expertise in numerical modelling so that we will only do as many experiments as are necessary to demonstrate fidelity of the neural simulations to predict the activation characteristics of a particular nerve/muscle/electrode configuration. In this case, we have a funded parallel program of experiments in human subjects that will inform, and be informed by, the animal work once we obtain ethical approval.

We collect whole muscles from the rodents after our interventions to change activity or loading and divide and store them purposefully to allow subsequent analyses such as epigenetic analysis or further biochemical analysis.

Most of our interventions to change activity or loading will use deuterated water to label newly-formed proteins so that we can follow protein synthesis and protein degradation by dynamic protein profiling .

We are working towards methods to follow adaptive changes in individual animals. This can be achieved non-invasively by imaging with ultrasound (already established in our lab)

and MRI which will be trialled at the imaging facility, and for which we need to retain the dual availability of this project licence.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Rats and mice with use of programmable implantable neural stimulators to change activity in groups of muscles within the hind limb:

This technique requires considerable technical input which we have achieved by close collaboration with a Medical Engineering Department. The alternative methods to produce resistance exercise in rodents are labour intensive and affect the daily routine for the subjects because of the need to arrange regular training such as weight pulling or ladder climbing. The programmed exercise that we use does not interfere with the daily life of our experimental animals once the implant is in place.

Furthermore, we activate only one hind limb. So the other acts as a contralateral control and reduces the need for separate groups of control animals. The stimulation devices are remotely programmable by radio frequency so that we can adjust the stimulus parameters to be just sufficient to cause activation of motor nerves without discomfort. We know this is possible from human experiments in which similar internal nerve stimulation can be adjusted to achieve motor activation without discomfort.

Other groups use systems so that great force must be used to access food. This system is not so well- controlled as our arrangement, and the exercise stimulus is spread over the feeding hours, and therefore not so obviously comparable to typical human exercise in purposeful sessions.

However most of the groups in this field make their data publicly available as we do, and we have published comparisons of our transcriptomic data with theirs, to make the best use of the various methods to approach the common research question to discover the cellular mechanisms involved in muscle growth and wasting.

The mice whose genome will be modified to investigate the effect of variations of the SIRT-6 enzyme using standard methods for transgenesis are not anticipated to suffer any deleterious effects as a result of that genetic modification. The mutation is one that is associated with increased health into old age in humans. The measurements we will use

to assess the effect of the genetic change on running ability, balance and motor control and heart function (ultrasound under anaesthesia) are designed to cause as little disturbance as possible to the daily lives of the mice.

Why can’t you use animals that are less sentient?

Some of our work, such as the testing of numerical predictions of interferential stimulation will be in terminally anaesthetised animals and we have good experience of the careful monitoring and adjustment of anaesthesia that such work requires.

There is no appropriately less sentient species in which we can investigate longitudinal changes related to exercise and disuse. There are some exercise models in zebrafish but given that the scientific community is concerned about the direct applicability to the human exercise response of results obtained even in the mouse compared with rat, we feel that continuation of experiments in mammalian species is justified.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have a continuous programme of refinement. Some recent examples include:

1: development of the mouse implant to minimise the size while retaining biocompatibility and reliability for up to 4 weeks implanted life. Testing of the implants in a simulated body environment (warm salty water) in the laboratory to ensure reliability once they are implanted.

2: Modification of the surgical procedure to reduce skin tension over the implant and thus improve skin integrity and wound healing. A change from Balb/C, an albino white strain of mice to C57/Bl6, a strain with slightly thicker, pigmented skin, has helped in this respect.

3: Switch to the F344xBrown Norway strain of rats. This crossed strain has a reduced incidence of ageing related debility, especially tumour formation in ageing cohorts than some other typical laboratory strains. It is not easily available so we have instigated a breeding programme specifically for this work.

4: Investment in a telemetric electromyogram (emg) system to allow recording of emg during silencing and recovery of common peroneal nerve from blockade with a drug derived from pufferfish (TTX) that causes temporary blocking of nerve signals when applied to a specific nerve. This will improve the interpretation of the effectiveness of the TTX block, and allow us to check whether a lower rate of infusion of the blocking drug is appropriate in older animals as we suspect.

5: Investment in new oxygen concentrators, thermostatic water body heaters and autoclave for operating theatre to ensure best practice during implant surgery and terminal anaesthesia.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Keep up to date with implant technology in published literature. NC3Rs newsletters

Contact and interaction with senior animal care and welfare officers and others who intentionally maintain high standards for training and responsibility for animal welfare.

We follow best practice to make sure that our experiments are planned, performed, analysed and presented in a way that provides the maximum amount of reliable data from each experiment. To this end, the ARRIVE guidelines provide excellent checks and balances and we are guided by them in all our planning. The PREPARE checklist is likewise useful and we foster open discussion to generate a culture of care in which to pursue our scientific objectives, discussing openly with our husbandry, administrative and research staff.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We maintain an email account at NC3Rs and stay abreast of development in alternatives, reductions and refinement.

We have weekly meetings with our collaborators to discuss the place of numerical simulation in neural activation as it applies to clinical practice in nerve repair and deep brain stimulation. Part of this project is to provide an evidence base to critically test some of the (sometimes unlikely) claims made for various applications of electrical stimulation.

We have regular open discussions in relevant committees including Animal Welfare and Ethical Review Boards concerning animal welfare.

77. Complement system in renal inflammation and bone development

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Therapeutics, Transplantation, Complement, Kidney, Bone

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?

Our aim is to develop new treatments (therapeutics) to prevent inflammatory blood proteins (complement system proteins) from causing damage to a transplanted organ. Secondly, these complement proteins can also influence bone development and we would like to investigate this to allow us to design therapies that promote normal bone growth 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 is important because it will benefit kidney transplant recipients as it will lead to better outcomes for them. In addition, our observations of abnormal bone development in the presence of a complement system deficiency will advance the development of therapies designed to promote normal bone growth.

What outputs do you think you will see at the end of this project?

Expected outputs:

New knowledge on the mechanisms of kidney transplant rejection and bone development.

Advance the development of medicines designed to inhibit the complement system at the time of transplantation.

It will provide clinical insight that could potentially lead to the development of diagnostic assays for detecting abnormal levels of complement proteins in the blood of patients that have an abnormal growth phenotype.

Who or what will benefit from these outputs, and how?

Observations of how complement proteins affect the normal development of bones and organs will benefit patients through development of therapeutic approaches. Therapeutics are currently in development within our laboratory. It is possible that these could form a component of clinical trials in humans within the next five to ten years.

The scientific community as a whole will benefit because our observations will have implications for the direction of research in other research laboratories across the globe, benefitting patients.

How will you look to maximise the outputs of this work?

Findings arising from this body of research will be presented at scientific and clinical conferences on an annual basis. In addition, colleagues will receive regular updates on progress, which will foster discussion on how the research aims can be modified and/or improved to advance our research.

Collaborations will form part of our research strategy; for example, we are currently collaborating with other institutions to determine sugar ligands for complement intermediates that are preferentially expressed on the surface of inflamed renal tissue.

Species and numbers of animals expected to be used

- Mice: 2600
- Rats: 210

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 laboratory is focussed on the role played by the complement system in mediating renal inflammation in the setting of whole organ transplantation. To this end, we have access to a number of genetically-modified mice that are deficient in a number of complement system proteins. We have been able to use these mice to determine the contribution of these proteins to the pathology of complement- mediated injury. Mice produce litters often and over a time frame that enables the execution of procedures in a timely manner, allowing the generation of new data relatively quickly. Mice will receive putative therapeutics via intravenous (IV) tail administration or IP administration, plus we have numerous complement-deficient strains (not available in rats). Commercially available rats will be used to test complement-inhibitory therapeutics and immunomodulatory reagents, when comparing to control-treated groups because the reagents can be administered directly to the donor kidney via the aorta (not possible in mice). Rats allow testing of efficacy in a larger rodent model.

Adult mice are used. The renal transplant procedure we have employed over the years would not be possible in younger mice. Typically, we use mice once they reach a weight of 20 grams or 6-8 weeks old. Rats would be used at between 12 to 14 weeks of age when they reach a weight of approximately 250g, which we have used in previous studies.

Typically, what will be done to an animal used in your project?

1. Donor tissue for transplantation

Purpose: to provide tissue for transplantation in other protocols. The donor animal (20 to 30 donors per year) will receive an optional dose of a therapeutic drug. Duration would be one day up to 3 months prior to excision of donor tissue. Administration of reagents will be performed in the absence of any form of anaesthesia. The animal will be put to sleep and an incision made in the abdomen so that donor kidney can be removed and placed on ice. The animal will then be killed by a schedule 1 method.

2. Renal transplantation with or without removal of the remaining opposite kidney

This procedure will be performed in either mice or rats and the purpose is to determine an effect on graft survival of the presence or absence of important proteins following renal transplantation. We anticipate 20 to 30 procedures per year. The transplant recipient of the donor kidney may receive a dose of therapeutic drug one day to three months before or after transplantation and will receive pain relief one hour prior to surgery. Renal Transplantation surgery: administration of general anaesthesia of recipient the and the left kidney is removed. After an incision in the abdomen, the donor kidney (see 'donor tissue for transplantation' described above), is implanted into the prepared recipient animal. The incision is closed, and the animal is given pain relief and then allowed to

recover in a warm box. Blood sampling may be performed at any stage by nicking of the vein with a needle. A urine sample may be collected during a period of no longer than 24 hours. Animals may be kept up to 15 days post-surgery. Removal of the remaining opposite kidney from the transplanted animal will be optional, between 5 to 10 days post-transplantation (AB). Animals will be killed by a schedule 1 method or by exsanguination under anaesthesia. Organs and tissues will be collected post-mortem for further analysis.

3. Renal ischaemia reperfusion injury

The animal may receive an optional dose of therapeutic drug, from one day to 3 months prior to induction of surgery. We anticipate 30 to 50 procedures per year. The animal will receive pain relief one hour prior to surgery. The animal will be put to sleep and an incision will be made in the abdomen.

Next, the kidney vein and artery will be clamped to prevent blood flow through the kidneys for a period no longer than 35 minutes. Following removal of the clamps, the abdominal incision will be closed, pain relief will be given to the animal and it will be allowed to recover in a warm box. Blood sampling by needle nick of the tail vein may be performed. Animals are typically kept for up to 15 days post-surgery (when we expect kidney function to have returned to normal) . Animals will be killed by either: i). a schedule 1 method or ii). by exsanguination under anaesthesia, completed by a schedule 1 method.

Organs and tissues will be collected post-mortem for further analysis.

4. Breeding and maintenance of GA animals - mild

The purpose it to produce and provide GA mice. Breeding and maintenance of genetically altered mice will be carried out by conventional breeding methods. An option will be to perform tissue biopsy to determine genetic status by one of the following methods: ear punch, blood sampling, hair sampling.

Rarely, due to technical problems during analysis, a second sample may be taken using the least invasive method. Maintenance of animals by methods appropriate to their genetic alteration will continue until they reach a maximum of 12 months of age. Animals not used on other protocols will be killed by a schedule 1 method, or by exsanguination under terminal anaesthesia, by a schedule 1 method. Animals not killed at the end of protocol will be used in another protocol under this or another project licence. Animals will be kept alive at the licensed establishment.

5. Breeding and maintenance of GA animals - moderate

The purpose is to produce and provide GA mice (double knockout [DKO]). Breeding and maintenance of genetically altered mice by conventional breeding methods. An option will be to perform tissue

biopsy to determine genetic status by one of the following methods: ear punch, blood sampling, hair- sampling. If removal of tip of tail is scientifically necessary, no more than 0.3 cm will be removed.

Rarely, due to technical problems during analysis, a second sample may be taken using the least invasive method. As an option, maintenance of animals by methods appropriate to their genetic alteration will continue until they reach a maximum age of 12 months of age. Animals not used for breeding and colony maintenance or on other protocols will be killed by a schedule 1 method; or by exsanguination under terminal anaesthesia, completed by a schedule 1 method. For animals not killed at the end of protocol, they will be subject to continued use in another protocol under this or another project licence. Animals will be kept alive at the licensed establishment.

What are the expected impacts and/or adverse effects for the animals during your project?

1. Animals for provision of donor tissue

We do not anticipate side effects from the biological agents or physiological carrier solutions used in the treatment of these animals, which are administered without general anaesthesia. Incidence of past technical failures for example, through internal bleeding, is nil.

2. Renal transplantation with or without recipient nephrectomy

We expect less than 5% of transplant recipients to display post-surgery signs of discomfort such as hunching, piloerection in the first 24 hours post-surgery. Kidney inflammation may lead to deteriorated renal function (a BUN of 50 mmol/L or over) and the animal will display clinical signs (such as piloerection, hunched posture, shivering, reduced mobility. We expect that less than 5% of transplant recipients will present with this level of renal functional impairment.

3. Renal ischaemia reperfusion injury

Animals are expected to make a rapid and unremarkable recovery from the anaesthetic within two hours. We expect less than 5% of animals to display post-surgery signs of discomfort such as hunching, piloerection in the first 24 hours post-surgery. We do not anticipate side effects from the biological agents or physiological carrier solutions used in the treatment of these animals.

4. Breeding and maintenance of GA animals - mild

Animals produced under this protocol are not expected to exhibit any harmful phenotype.

5. Breeding and maintenance of GA animals - moderate

Approximately 30 to 35% of animals produced under this protocol are expected to exhibit harmful phenotypes, presenting with spinal deformity indicative of scoliosis. This phenotype however is not visible until 13 to 15 weeks of age. We aim to use all mice under this protocol at 8 weeks of age when the phenotype is not visible. Affected mice groom, feed and drink normally and appear to have no associated behavioural deficiency.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice
Sub-threshold 80%
Mild 50%
Moderate 25%
Non-recovery <1%
Severe 0%

Rats
Sub-threshold 80%
Mild 10%
Moderate 10%
Non-recovery <1%
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 studies outlined will test the efficacy of therapeutic inhibitors of kidney inflammation during transplantation. We envisage that these reagents will be applied in the human transplant setting. The mice and rats have intact immune systems similar to humans. Accordingly, inhibitors will first have to be tested in live animals to show whether protection fro m transplantation inflammation is achieved and can ultimately be translated to the human setting.

Which non-animal alternatives did you consider for use in this project?

Complement inhibitors will be tested in vitro, on cultured renal cells, or in a specialised experiment, to determine the dose of inhibitor for subsequent testing in animals.

Why were they not suitable?

Once the efficacy of an inhibitor has been demonstrated using these in vitro models discussed above, testing in live animals will be required. The use of animals is necessary because ultimately, we would like to apply the use of inhibitors of kidney inflammation in human patients – animals are required as a first step as the biology of mice and rats will mimic what we expect to observe in the human transplant 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?

Number of mice estimate includes breeding of mouse colonies over the 5-year period of the licence. The number of rats is based over a 5-year period. Rats will be purchased from external suppliers and the estimate represents a maximum we envisage being required for kidney inflammation injury studies.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Due to good reproducibility in the transplantation technique, we envisage that only small numbers of control and therapeutically inhibited animals will be required. For transplantation studies, we envisage 4-6 recipients in each experimental group (based on previous studies in our laboratory) , namely inhibitor-treated groups and a control groups. For kidney transplants, one donor animal will provide one organ (the left kidney), for transplantation into one recipient. The contralateral kidney cannot be used as it is difficult to access, being partially covered by the liver. The left kidney is more straightforward to expose and remove. Within our department we have a dedicated statistician who provides advice when designing in vivo studies. Calculations have been performed on in vivo study designs to ascertain the smallest number of animals required for a statistically meaningful result to be achieved.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Mice will be bred at the animal facility for studies. We aim to keep the number of breeding pairs to a minimum and monitor the age of offspring closely, such that procedures are performed on offspring as they reach the correct age/weight. Some mice from mild breeding and moderate breeding protocols (protocol 1 & 2), may be shared with other research groups after schedule 1 killing for tissue/cell extraction.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mouse and rat models of kidney injury are to be employed in this project. Careful monitoring of treated animals, keeping them warm and with easy access to wet food, and administration of pain relief, ensures that their suffering is minimized. Refinement: We have a range of well-characterized mice that are deficient for proteins associated with kidney inflammation. All donor tissue is obtained when the animal is under terminal anaesthesia. The anaesthetic method we employ using isofluorane combined with analgesia and a warming pad, significantly reduces mortality and recovery time following surgery in our kidney injury models - mice recover from this anaesthesia route more rapidly than application of injectable anaesthetic, ensuring a return to normal core body temperature quickly, thereby reducing the chance that the animal may suffer from hypothermia. Mice will be under anaesthesia for short periods of time: recipient typically 40 minutes for the renal transplantation procedure and up to 35 minutes for a non-transplant kidney injury procedure. Strict asepsis (meeting at least the standard set out in the Home Office Minimum Standards for Aseptic surgery), will be observed throughout all surgical procedures. We will avoid any post-operative discomfort by regular monitoring of animals (daily and with assistance of BSU staff) and providing pain control in the form of exogenous analgesia (perioperative and post-operative analgesia as advised by the NVS will be administered). If the animal looks sick (hunched, lack of grooming (dull coat with visible piloerection); slow movement; dry eyes), the relevant humane endpoints as detailed in the protocol will be adhered to. The NACWO and/or NVS may also be consulted and if the humane endpoint is reached, the animal will be humanely killed by a Schedule 1 method. The anaesthetic method we employ using isofluorane combined with analgesia and a warming pad, significantly reduces mortality and recovery time following surgery in ischaemia/transplantation models - mice recover from this anaesthesia route more rapidly than application of injectable anaesthetic, ensuring a return to normal core body temperature quickly, thereby reducing the chance that the animal may suffer from hypothermia. In our models, determination of BUN (Blood Urea Nitrogen), is routinely us

ed as a standard endpoint (≤ 50mmol/L; the normal BUN range is 5 - 10 mmol/L). At a BUN of 50 mmol/L the kidney function will continue to deteriorate rather than improve. This signals that overt clinical deterioration will ensue, therefore the animal will be humanely killed by a Schedule 1 method.

Why can’t you use animals that are less sentient?

Due to the size of the animals, we require that they are to be used at the adult stage of life. Mice are too small to operate on below 19 grams in weight. We would like to use rats as they constitute a larger rodent model in which we can directly administer inhibitor via the aorta directly to the kidney. The transplantation model of complement inhibition would be carried forward to the clinic in adult humans too.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

To minimize harms to animals that have had a regulated procedure, they are provided with analgesia, peri-operatively and post-operatively. They are housed singly in a warm box to aid their recovering and provided with wet mash food at floor level so they do not have to exert themselves to reach their standard feed tray, for which they would have to stand on their hind legs. They are monitored closely for signs of distress such as piloerection and hunching in the first few hours post-surgery and two to three times a day subsequently and weight is measured to ensure they are feeding adequately.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

I would follow the ASPA code of practice. The 3R's Resource Library also provides good guidance. PREPARE guidelines and ARRIVE guidelines will also be adhered to.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

By regularly visiting the website (https://www.nc3rs.org.uk/), for regular updates. In addition, we receive regular bulletins from Biological Services. In addition, colleagues working in the field of transplantation research are a rich source of information for development of refinement methods.

78. Studying protein homeostasis in the context of healthy and malignant stem cells

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
- 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

Stem cells, Cancer, Therapy, microenvironment, systemic stress

Animal types	Life stages
Mice	neonate, juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to study normal stem cell development and understand how these processes change during malignancies, such as cancer (principally, but not exclusively, focused on the blood system). We will also investigate how healthy and unhealthy cells interact with different tissues and how we can identify and target unhealthy cells whilst preserving healthy tissue. Lastly, we aim to translate our basic laboratory research into clinical benefit.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Our bodies produce an estimated 4-5 billion new blood cells every day, required for numerous essential functions including immunity, oxygen transport and clotting at sites of bleeding. The system of stem cells and their progeny in charge of producing these cells is known as the haematopoietic system, with haematopoietic stem cells sitting at the top and being responsible for life-long blood production. The system declines with age, and eventually fails. This can be accelerated by accumulation of mutations leading to cancer (leukaemia), including Acute Myeloid Leukaemia (AML), a disease of the blood system with one of the poorest prognosis, and very few treatment options.

This project licence will aim to better define both healthy blood stem cells, their progeny and their cancerous transformation leading to leukaemia, as well as investigating how this occurs in other tissues and how it affects blood cell development. To do this it will focus on how proteins are regulated within healthy and malignant cells to understand their nature and develop new strategies to selectively kill cancer cells whilst preserving and even boosting healthy cells. Ultimately, this will lead to studies aimed at improving the life of patients suffering from AML, as well as other diseases, and have a major impact on our understanding of stem cell development.

What outputs do you think you will see at the end of this project?

This project will improve our fundamental knowledge of how stem cells are regulated by proteins under a variety of conditions to ultimately provide new targets and therapeutic approaches to aid health and combat disease.

By using different mouse models, stimulatory and malignant transformation conditions, we will produce research publications providing in depth knowledge of:

1. How proteins vary in healthy blood stem cells, their progeny, and how this is important for maintaining day-to-day functions.

2. How blood stem cell proteins respond to stresses such as infection, bleeding and chemotherapy.

3. What role proteins play in the malignant transformation to cancer in the bone marrow and across tissues.

4. How to improve the production of stem cells without the use of animal models.

We will use the information gained in these studies to design new ways to combat diseases such as cancer, or bone marrow failure following chronic infection, to design new therapeutic approaches to improve:

1. The targeting of drug resistant cancer cells of the blood system, whilst simultaneously preserving healthy tissue and reducing the toxic burden of chemotherapy.

2. Recovery of the blood system post infection, chemotherapy or bleeding.

3. Production of blood stem cells in a dish for regenerative medicine purposes.

We also anticipate that throughout our studies we will uncover new processes and pathways that will be relevant to improve understanding of variation, modelling of stem cell systems without animals (Replacement in the case of human stem cells) and improved animal model development to study multiple systems simultaneously (Reduction/Refinement).

Who or what will benefit from these outputs, and how?

The initial beneficiaries of this work will be the scientific communities working across blood, stem cell and protein research. These researchers will benefit from the large amount of data we will publish open access and make freely available through our institute website.

In the medium-term, this work will benefit the scientific community interested in generating new animal models that better support studies of healthy and malignant blood stem cells.

In the long-term, this work will benefit patients:

- With cancers treated with chemotherapy as it will design new ways to reduce toxicity.

- With blood cancers which are resistant to classic chemotherapeutic approaches.

- Who require bone marrow transplantation, gene therapy or blood products for improved in-patient care.

How will you look to maximise the outputs of this work?

Outputs of this work will be communicated by publication in open access journals to make all of our results freely available to the scientific community. In addition, our group will present our findings at national and international meetings to disseminate the knowledge as widely as possible. We will also engage with local patient advocacy groups to discuss our work and get feedback from the general public, including the haematological research network (HMRN.org) local patient advocacy groups.

In addition to these engagement processes, we will endeavour to make our analysis pipelines, protocols and primary data available through our University web portal. This will

allow researchers to interrogate our protocols and data and help inform their practices. All currently used mouse models are available to the wider scientific community and any further models we may generate through these studies will be made available after peer-reviewed publication.

Species and numbers of animals expected to be used

- Mice: 13,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Animal work is required to address the specific scientific questions proposed because it is not yet possible to reproduce the complexities of the haematopoietic stem cell (HSC)/leukaemic stem cells (LSC) environment outside of mice (in vitro), nor the changes taking place during cancerous transformation across tissues and cell types. Additionally, the bone marrow microenvironment (bone marrow niche) and movement of stem cells to other organs (extramedullary haematopoiesis) can only be studied in vivo (in mice). The mouse is the smallest and least sentient vertebrate that can be used for these experiments.

The mouse haematopoietic system is extremely similar to the human, with many findings obtained from mouse studies directly translated to humans, and we will use mouse life stages representing both adult and paediatric cancers (neonatal, juvenile and adult mice). Moreover, the availability of a large number of genetically modified mouse strains makes this species ideal for the scientific approach used in this project.

Whilst the majority of the data will be generated with in vivo models, multiple approaches will be used to validate findings in vitro before large scale in vivo tests.

Typically, what will be done to an animal used in your project?

We will use animals to study the effect of genetic engineering and/or external challenge on stem cell biology and on blood cell development. In some cases mice will require treatment before stem cell transplantation, which may include irradiation or chemical treatment.

Mice may be treated with agents that induce cancer or inflammation or other external stresses. These could be given via drinking water, food or injected: in a vein, the mid-torso cavity, directly into the shin or thigh bone, into the nose or under the skin.

During protocols mice may have bone marrow or peripheral blood sampled at regular intervals to study effects of different stresses. Blood sampling is mild and can be carried

out on conscious mice, but bone marrow aspiration will be carried out under anaesthesia. Mice may also be subject to total body imaging

Experiments will vary in duration from days (e.g. initial responses to cytotoxic therapy) up to months (e.g. adoptive transfer of blood stem cells and monitoring up to 24 weeks).

What are the expected impacts and/or adverse effects for the animals during your project?

Some mice will be expected to grow tumours, which could lead to weight loss, anaemia, loss of coat condition or loss of mobility in accordance with development of a tumour. Mice undergoing transplantation (injection of stem cells) may experience moderate severity at early stages, especially immunodeficient mice (genetically engineered mice without a competent immune system), for whom typically in around 10% of cases, animals might show signs of being unwell in the first 10 days after conditioning. This would include loss of coat condition, minor weight loss (<10%) and reduced mobility.

Injection into the bone marrow cavity is not expected to have adverse effects except for on rare occasions where immunodeficient mice may fail to recover after anaesthesia. When a drug delivery device is implanted, there is likely to be some post-operative pain and risk of infection. Chemotherapy may cause multi-organ toxicity to some extent (e.g. loss of full gut function leading to rare/short term cases of diarrhoea, or mild necrosis of spleens seen as small black sections at the end of the spleen at autopsy), but this will be mitigated by new therapies developed in this licence. These symptoms are usually transient, and most mice recover after a few days.

When imaging mice, physical restraint and anaesthesia can affect an animal's general well-being when carried out for prolonged periods of time.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

For all immunocompetent mice they are expected to be 80% mild and 20% moderate.

For all immunodeficient mouse experiments they are expected to be 50% mild and 50% moderate.

This licence will use immunocompetent mice approximately 75% of the time. Therefore the overall severity would be 72.5% mild and 27.5% moderate severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

It is not currently possible to study blood stem cell self-renewal, drug sensitivity and full differentiation potential using cell culture alone because the methods developed thus far cannot discriminate a stem cell from a multi-potent progenitor. As such, the only method currently available for the identification of blood stem cells is repopulation after transplantation into conditioned mice; however, the significant amount of data we will generate from proteomics experiments will aid in developing biomarkers, culture systems and reporters to screen for candidate regulators using cell culture methods in the future.

In order to study drugs that target blood cancer development, an in vivo whole animal model is needed. In particular, since haematological cancer development is a complex process that involves interaction of the tumour cells with the local environment and the immune system, it is not currently possible to recreate these interactions in a dish.

Finally, this programme of work has a specific aim to develop new animal-free culture conditions to assess both mouse and human blood stem cells under homeostatic conditions.

Which non-animal alternatives did you consider for use in this project?

Within this project we will be testing new hypotheses derived from in vivo experiments to generate new ex vivo co-culture (culture of cells together in a dish) systems for both human and mouse stem cells.

Therefore, we will use ex vivo co-culture systems to complement in vivo work and, in some cases, use these systems as pre-screening tools to reduce the number of animals used and develop and validate non-animal alternatives in the future.

Why were they not suitable?

We cannot exclusively use ex vivo co-culture systems, as the most robust scientific model (in terms of real-world translation) remains in vivo transplantation and drug responses, therefore the two experimental approaches will complement each other.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have previously used all the experimental methods proposed in this project licence and animals are estimated as per our previous work. Prior knowledge of our genetically engineered murine models allows us to predict how many mice to breed to obtain numbers for our experiments. We have used data from our previous experience in statistical analysis to inform us of how many mice are required for individual experiments. Example estimation of animal numbers are detailed in technical protocols and would be 9,000 immune competent mice and 4,000 immunodeficient mice.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Standard operating procedures and data analyses are continuously optimised to use the fewest number of animals possible. When designing the experiments, we perform statistical analyses (e.g. power calculations) to ensure that we use the minimum number of mice per group that will be informative. We have stated group sizes and experimental approaches in our previous publications, which all follow ARRIVE guidelines for the reporting of our animal experiments in publications.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Initially we will use human cell lines (including patient derived cell lines) to study the biochemistry of different protein regulatory pathways before transferring to in vivo experiments.

To initially screen for putative populations of HSCs/LSCs under different selection conditions or treatments, we will use cell culture assays to first test for potential effects prior to full in vivo assessment.

In the case where the correct genetics of experimental mice occur less frequently than normal healthy mice tissues will be biobanked for future use.

Low input mass spectrometry currently under development will allow reductions in the number of animals because it will allow the collection of more data from each animal and reduce the need to pool multiple replicates. Additionally, we adopt a barcoding system (similar in principle to that used in supermarkets), which both blinds sampling at the analysis stage and provides equal sampling of each datapoint without bias, as per our previous publications.

A main focus of our work is studying healthy and malignant cells side-by-side and thus we will carry out experiments studying effects on healthy and malignant cells in the same animal using so-called chimeras (mice with cells from two different origins) where appropriate. This will reduce the requirement of separate studies on healthy and malignant 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 wild-type (WT) and genetically modified models most faithfully represent the disease status seen in patients (iAML) and the unbalancing of protein regulation (Cks1/2) critical for our investigations.

We will use irradiation and busulfan (chemical) conditioning for creating haematopoietic chimeras. Irradiation will be administered as a split dose to immune-competent mice to reduce the severity of the procedure as per our previous publications and busulfan conditioning will be first tested in dose escalation studies in all models to ultimately replace irradiation in future projects as it is considered a more clinically relevant conditioning regimen and less toxic to mice.

We will also use c-Kit mutant W41 mice as reconstitution recipients where possible as these require only a single sub-lethal irradiation dose and do not require carrier cells from an additional mouse.

For drug/small molecule/systemic challenge testing we will use dose escalation studies against either WT or chimeric mice to initially estimate a suitable dose in pilot experiments before carrying out larger experiments.

When animals start to show any of the specific symptoms relating to pain from the tumours or treatments they may be given analgesia that is not likely to influence the experimental outcome.

Why can’t you use animals that are less sentient?

Animal work is required to address the specific scientific questions proposed because it is not yet possible to reproduce the complexities of the HSC/LSC niche in vitro, nor the changes taking place during cancer development. The mouse is the smallest, least sentient and most appropriate vertebrate that can be used for these experiments. The mouse haematopoietic system is extremely similar to the human, with many findings obtained from mouse studies directly translated to humans. Moreover, the availability of a large number of genetically modified mouse strains makes this species ideal for inducible oncogene transformation and cell surface-based isolation and analysis as proposed in this project, a key reason why we cannot use other, less sentient models (e.g. zebrafish).

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

For irradiation, we will use split dose on WT mice and systematically test the replacement of irradiation with busulfan conditioning (in cases where we haven't used it before) to be both more clinically relevant and less harmful to mice.

For transplantation protocols, we follow LASA guidelines including the use of the smallest needles possible and put pressure to encourage haemostasis after collection/injection. We are currently re- designing our animal restraint device to allow firm but sympathetic restraint. For injections we immediately return mice to cages with soft food within easy reach and monitor mice closely for 24 hours and if significant signs of distress are noted pain killers will be administered.

For drug/small molecule/systemic challenge testing pilot dose escalation studies minimise the welfare costs by reducing the number of animals tested initially before widening to a larger cohort when the optimal dose is found.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All experiments are planned based on the "Guidelines for the welfare and use of animals in cancer research" by Workman et al., Br.J.Cancer (2010) and the ARRIVE v2 (Animal Research: Reporting of In Vivo Experiments version 2) guidelines set out by the NC3Rs. For surgical procedures, we follow the LASA guidelines on aseptic procedures (LASA 2017 – Guiding principles for preparing for and undertaking aseptic surgery) and for drug dosing we also follow LASA guidelines (including pilot studies and dose escalation).

Longitudinal AML monitoring experiments are planned according to the principle of refinement, for example multi-site monitoring will maximise the amount of information obtained from each mouse. Moreover, single cell in vitro functional assays will allow generation of large datasets with 100s of data points from individual mice (refinement).

Finally, rather than large scale testing of multiple models, we will generate initial data from one model and from these experiments we will carry out targeted mouse experiments using alternative models to validate our findings. This will reduce the number of models and different tests required (reduction), and provide a clear strategy for LSC targeting and HSC recovery under multiple conditions (refinement).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We are continuously reviewing our techniques and monitoring new developments that lead to a refinement of regulated procedures. We have regular 3Rs update meetings with our animal welfare officer internally, receive regular updates with regards any advances in the field from the facility manager. Moreover, the PPL regularly takes part in 3Rs training

courses and is updated on 3Rs related policies. In addition, one arm of our project is aimed at collecting biologically relevant data to design new systems to replace mice in research. Therefore, we will be attending conferences, staying up to date with the literature and constantly reviewing our protocols to improve our work.

79. Studying novel treatments for ovarian 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

Ovarian cancer, chemotherapy, immunotherapy, Biological therapy

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of the project is to identify new drug combinations for treatment of ovarian cancer that can target cancer cells as well as other cells in the tumour microenvironment such as stromal, endothelial, immune cells and others which can improve response to chemotherapy, prevent or delay relapse.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

High-grade serous ovarian cancer (HGSOC) is the most common type of ovarian cancer. Around 7,000 new patients are diagnosed every year. It is the most lethal gynaecological malignancy. Over 80% of the patients are diagnosed at an advanced stage when the cancer has already spread in the abdomen. Treatment is usually a combination of chemotherapy and surgery. However, 75% of the patients will suffer from relapsed disease within 2 years of diagnosis. Treatment of relapsed disease is even more challenging. Patients who responded initially will eventually develop drug resistance with hardly any treatment options. In the past 20 years, PARP inhibitors were nearly the only effective targeted therpy recently added for treatment of ovarian cancer. These are a group of drugs that inhibit PARP enzyme. PARP (poly-ADP ribose polymerase) is an enzyme found in all cells to help damaged cells to repair themselves. PARP inhibitors stop the PARP enzyme from doing its repair work in cancer cells and so they eventually die. They are more effective if the BRCA genes, which are also responsible for the cell repair, are mutated and not functioning as this will further prevent the cancer cells from repairing themselves. PARP inhibitors improved survival mainly in patients who have tumours cells with defective repair mechanisms (such as BRCA gene mutation). Those patients represent only 20-50% of the ovarian cancer patients so are not suitable for everyone.

Cancers are not just collections of malignant cells but complex tissues that contain many normal cells and molecules that are recruited and corrupted to help cancer grow and spread. Over the past 10 years or so, scientists and clinicians have learnt that treatments that alter the behaviour of non-cancer cells such as immune cells and blood vessels can have major pro-tumour or anti-tumour effects. These types of treatment are called biological therapies and immunotherapies. So far, ovarian cancer research has not been successful in finding effective biological therapies except for bevacizumab which inhibits the formation of new abnormal tumour blood vessels (angiogenesis). Bevacizumab has just 2.5 months improvement in survival. There is no effective immunotherapy so far. Hence, there is an unmet clinical need for treatment of primary and relapsed HGSOC.

So far, there have been a great advance in human in vitro 3D multicellular models. Those models have the advantage of being built from human tissue so very close to human disease. However, they contain few cell types and not as complex as the human tumour, it is also not possible to keep them alive in culture for more than 2-3 weeks. Furthermore, currently, they cannot answer the most important question about a new drug or drug combination which is the survival advantage of using this drug.

Hence, the importance of animal models. This complexity of the tumour microenvironment can best be reproduced via in vivo systems such as mouse models of cancer. In our lab, we are implementing a joint platform of human 3D multicellular models to screen for eligible compounds then promising compounds will be tested in mouse models of ovarian cancer. This project aims to test promising compounds, identified from our in vitro models and large data analysis on patients' samples, on survival and relapse in mice which cannot be answered via the in vitro models.

What outputs do you think you will see at the end of this project?

At the end of this project, we aim to obtain new knowledge about the tumour microenvironment of ovarian cancer, both primary and relapsed disease. We also aim to find new immunotherapy and biological treatments, single, combination or sequential, that can cure tumours and delay relapse. This information can potentially be translated to new clinical trials.

We will publish our work in international journals and present our work at national and international meetings (In the past 5 years there have been 2 publications that have resulted from work I carried out completely, or partially in collaboration with others and one paper is currently under revision).

Finally, we will make all the bioinformatics data available on publication which can be used by the scientific community who showed excellent interest in the models that will be used on this license.

Who or what will benefit from these outputs, and how?

Patients with cancer - we aim to take successful treatments for clinical trials, based on our results, in advanced ovarian cancer by the end of this project.

Scientists and clinicians, who study the tumour microenvironment from reading our publications and from listening to presentations at meetings.

Ovarian cancer researchers, who can use our publicly available ovarian cancer models and data sets.

How will you look to maximise the outputs of this work?

Collaboration is an important way to maximise our outputs. For instance, we have international collaboration with other groups working on mouse models of ovarian cancer that are fully characterised and we exchange the cell lines and data to broaden our data sets and choose the best model that can answer our questions. We also have collaboration with pharmaceutical companies who provide us with new compounds with optimal doses and known side effects to test in our ovarian cancer models.

We will publish successful as well as unsuccessful approaches and, importantly, we will make clear similarities and differences between our mouse cancer models and the human tumour microenvironment that may influence our interpretation of the data. We also make our bioinformatics data publicly available on publications.

Species and numbers of animals expected to be used

- Mice: 3000 adult females

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Our work is entirely based on mouse models of cancer that most closely replicate the human tumour microenvironment. The mouse model we will be using is ovarian cancer model. We have several ovarian cancer cell lines of murine origin. So, all the mice that will be used on this licence will be adult females.

Typically, what will be done to an animal used in your project?

The most common type of experiments conducted on this license will involve injection of ovarian cancer cell line intraperitoneally to induce tumour development in a way similar to the metastatic disease that develops in patients. Tumour growth will be typically detected clinically by abdominal palpation or the development of ascites. Mice will then receive one or more drugs (or placebo) for a specific number of weeks, depending on the drug given, to study the effect of the drug or drug combinations on tumour growth. Mice may be culled after certain pre-defined number of treatment cycles to harvest tumour tissue. Analysis of tumour tissue will be performed to study the mechanism of response or resistance to the tested drugs. Alternatively, mice may be monitored closely until they are culled at humane endpoint. The effect of different drugs or drug combinations on prolonging life will be compared to see the survival benefit of a new drug or drug combination. When mice are culled, tumour tissue will be harvested for further analysis as above.

Treatment will be given in a way similar to how it will be given in patients. In some of our experiments, we will use chemotherapy as a means of treating cancer. The minimum number of cycles of chemotherapy will be administered, typically three or six cycles (+/- 2 consolidation cycles), depending on the model. This will generally be given by abdominal injection, once weekly. Other treatments, such as biological therapy or immunotherapy, will typically be administered concomitantly or sequentially.

We may give treatment orally (generally once/day) or by abdominal injection (generally once/twice weekly). In slower growing tumour models where we see a therapeutic effect with certain biological therapies and provided there is no adverse effect, we may continue treatment for a maximum total period of 22 weeks. Extended treatments will only be carried out where there is a clinical benefit to the mice.

Some animals may be re-challenged with tumour cells injected in the abdomen. This experiment will be carried out rarely. Imaging will confirm that the mice are tumour-free before the tumour cells are re- injected. The aim of this type of experiment is to determine whether the mice have developed an anti- cancer immune response during the first course therapy, and that this will prevent tumour growth when mice are re-challenged. Mice will

not receive a therapy after the second challenge but will only be monitored for tumour growth according to our standard protocols.

In one of our mouse models develop relapsed disease after good initial response. Mice receive cancer cell injection intraperitoneally then treatment with chemotherapy for 3-6 cycles. Mice will then be left to develop relapsed disease. Treatment with one or more drugs will be given to treat relapsed disease and study the effect of the new drug treatment on curing relapsed disease and prolonging life. The total treatment period will not exceed 22 weeks.

Finally, small pilot drug tolerance studies may be conducted to ensure the tolerance of the mice to a new previously untested drug combination before embarking on one of the above four main experiments.

Imaging may be incorporated into any study carried out in this protocol and will generally be used to confirm the presence or absence of tumour or to monitor/follow up tumour growth and treatment response. This would also reduce the number of mice we use, as it will allow us to study, in the same experiment, short term and long-term effects of a therapy without the need to cull the animal.

A blood sample may be collected to monitor the animal's health or the effect of the treatment. Generally, we would expect to run these checks up to once weekly over the course of an experiment. A volume of twenty to fifty microlitres is sufficient for this purpose.

What are the expected impacts and/or adverse effects for the animals during your project?

From our past experience, we predict that the planned studies will cause only mild or moderate discomfort. Animals are expected to return to normal activity within minutes after treatments such as cell injections or drug administration. We do not expect adverse reaction related to drug treatments or their administration but the repeated handling of the mouse for dosing is likely to cause some transient distress. Some distress is likely to occur in the advanced stage of tumour growth which could interfere with normal bodily functions. Indications of moderate distress include; weight loss up to 15%, reduced food and/or loss of appetite, changes in activity and /or posture such as hunched posture. Humane end point will be applied for those 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)?

All the animals used in this project, except those used in the small pilot drug tolerance studies, will be injected with cancer cell lines to induce tumour growth then will be subjected to single agent or combination therapies (or placebo) with the aim of curing the cancer and preventing relapse. In some experiments, animals may undergo some form of imaging under anaesthetic to assess their tumour burden. For these reasons, the procedures will be of moderate severity. All the animals, 100%, will be subjected to moderate severity even if they don't receive the cancer cell line injection (as in the pilot studies) because they will still receive nearly daily treatment of combination therapies.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 because:

1- The complexity of the tumour microenvironment including malignant cells, stromal, vascular, lymphatic, and different immune cells in addition to other normal cells is impossible to model in any in vitro model. We currently have 3D models that can hold up to 5 different cell types only.

2- Currently, the in vitro models are not able to answer important questions that are clinically relevant such as the efficacy of certain drug or drug combination on prolonging survival and progression free survival and preventing tumour relapse.

3- It is not possible to study the dynamic effect of a particular treatment in patients due to limited chances for biopsies in patients that are limited to only at time of diagnosis and surgery so using good mouse models enable studying this dynamic effect and response development.

Which non-animal alternatives did you consider for use in this project?

Collaborators have developed invitro multicellular human models that can hold up to 5 cell types for up to two weeks. The complexity of those models will never recapitulate the complexity of the actual tumour in patients or mice. However, we use these models to screen compounds and select the promising ones for testing in vivo in the mice. This approach helps to minimise the use of mice to only a few limited promising compounds. Mouse experiments will then be used to study the translational potential of those compounds on mice survival and relapse.

Why were they not suitable?

The multi-cellular models cannot replicate the cellular complexity of the mouse cancer models, nor can they be grown over the longer periods of time needed for some of our experimental treatment schedules. They are also not suitable to study drug combinations. They cannot be used to fully answer clinically relevant questions related to cancer survival and relapse, which are the most important parameters used in clinical trials.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

I have been working on ovarian cancer mouse models for the last five years. The estimated number is based on my experience and knowledge of the natural history of the mouse models we use and experiments (published and unpublished) that have given us statistically significant results. We have extensive experience of murine cancer models, and over the past 5 years have collected data on the most suitable models and number of animals per group to generate reliable data. The studies in this project will utilize these models while advancing our work to improve on therapies. We are now more focused on the interactions of our novel therapies with the tumour microenvironment and the host immune system. We estimate up to 3000 animals will be sufficient for testing efficacy of various combination therapies (including novel therapies and conventional therapies). However, these are the maximum estimated number of animals for the duration of the project license and in each study an exact calculation will determine the numbers needed to achieve statistical significance.

We will also refer to the NC3Rs Experimental Design Assistant: https://www.nc3rs.org.uk/experimental-design-assistant-eda

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Mouse models are part of cancer study platform in which we have in vitro human multicellular models that can hold up to 5 different cell types and ex vivo human ‘tissue slices’ taken from human tumours for live imaging. This is in addition to large data generated from studying the tumour DNA (genomics) and RNA (transcrioptomics) from both human and mouse models. These sets of information and human models enables hypothesis generation and drug screening then only the promising ones will be tested in mice. The impact of all these models can be seen as we have no breeding aspect on this license.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

As we now have extensive data on the molecules, cells, and extracellular matrix of ovarian cancer, we can first conduct computer-based studies and work in the other human tumour microenvironment models in the lab before moving to the mouse experiments. This can be followed by small pilot study in a mouse model. Small pilot studies will be very important in terms of reduction, especially when we are assessing combination therapies that were not tested before by collaborators or in the literature. These small studies will help flag intolerance issues before embarking on big 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 are studying the ovarian cancer microenvironment therefore we need mouse models that develop cancers in the correct microenvironment. These are called orthotopic tumours. For the ovarian cancer models, we inject the cancer cell lines into the peritoneal cavity then start treatment on established tumours. We inject the cancer cells intraperitoneal to mimic the ovarian cancer spread in patients. It is not possible to inject directly in the ovaries because the ovaries in mice are surrounded by an ovarian bursa which may act as mechanical barrier that hinders the spread inside the peritoneum. We continue to seek improvements to our techniques; this includes using imaging techniques to early detect the tumour and start treatment as appropriate and reduce the number of mice.

Why can’t you use animals that are less sentient?

We cannot use animals that have been terminally anaesthetised because some of our models evolve over several weeks and months. We try to replicate, as closely as possible, human cancers that generally grow slowly. Also, tumour microenvironments do not develop in days; it takes time to recruit the other cells and for important components such as extracellular matrix and blood vessels to develop in cancers.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

One of the ways to minimise harm is to refine our non-invasive imaging methods. This means that we will be able to find out at a very early stage when a tumour is initially growing or has started to spread somewhere that will cause increased suffering. We are collaborating with a number of imaging scientists to refine our experiments in this way.

For animals needing daily regular treatment course, handling and disturbance of the mice are kept to minimum where mice get checked (+/- weighed) and treated at the same time to avoid multiple disturbances.

Also, for mice needing dosing via oral gavage, sugar-based diluent as a ‘reward’ is included, when possible, if it doesn’t affect the drug preparation.

Mice are also started on wet mash diet to enhance their general condition and also given as a reward after treatment procedure.

Regular involvement of the NACWOs to be alert of any potential welfare concern arises. Engagement with the animal unit staff is crucial to best culture of care practices in these studies.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

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How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We have an effective 3Rs network through the NC3Rs and attend our establishment 3Rs events as well as those within our network and LASA. Staff will be attend conferences (such as the IAT Annual conference) and will bring back best practice in terms of the 3Rs and new techniques and we will keep abreast of the literature to ensure that we use the best and most reproducible methods.

An example of an advance highlighted to us by NC3Rs and our resident Named Veterinary Surgeon which we have implemented is tunnel handling and new method of scruffing before giving treatment. Such procedures have had a noticeably positive effect on reducing animal stress and anxiety prior to carrying out procedures and during serial dosing of animals.

80. The biology, detection and treatment of lung cancer & mesothelioma

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Lung Cancer, Mesothelioma, Inflammation, Cancer immunology, Cancer treatment

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 investigate how genetic mutations, either alone or in combination with inflammation, drive lung cancer and mesothelioma (a cancer of the cells lining the chest cavity and chest organs) development, progression from benign to malignant disease, and to test new treatment strategies based on our findings. A key aspect of this work is to develop a deeper understanding of how the immune system interacts with developing tumours to either promote or fight back against 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?

Cancers of the lung remain the number one cause of death by cancer worldwide. Although many lung cancers are widely recognised to be caused by smoking, a growing number of lung cancers are linked to air pollution. Mesothelioma is a type of cancer typically arising on the outer lining of the lung, or the inner lining of the chest wall, following inhalation of asbestos fibres. The UK currently has the highest incidence of mesothelioma worldwide and new cases continue to arise in the UK, from both work- related and environmental exposure to asbestos.

Smoking, air pollution and asbestos are all known to provoke chronic inflammation, however, our understanding of how this chronic inflammation contributes to cancer is very poorly understood. Both lung cancer and mesothelioma respond well to a type of treatment called immunotherapy, which allows the body's own immune system to fight back against the cancer. However, effective responses to immunotherapy are highly variable in both cancers and the reasons for this are also very poorly understood.

What outputs do you think you will see at the end of this project?

A better mechanistic understanding of how inflammation contributes to cancer development and progression to deadly disease

A better mechanistic understanding of how cancers hide from the immune system

A better mechanistic understanding of how specific cancer mutations and cellular control systems interact with inflammation and/or contribute to evasion of the immune system to enable cancer progression

Pre-clinical validation of multiple new cancer treatments to "de-risk" future clinical trials

New genetic tools to empower and improve scientific investigation by other research laboratories

- Multiple peer-reviewed publications in well-regarded scientific journals
- Multiple presentations at international cancer conferences and pre-print publications to enable rapid dissemination of key findings ahead of formal peer-reviewed publication

Who or what will benefit from these outputs, and how?

Short-term: New insights generated over the course of this work will substantially advance scientific understanding of how lung and chest cancers interact with particle-driven inflammation and the immune system. These will enable ourselves and other researchers to find new ways to treat these deadly cancers. Additionally, we collaborate extensively with other researchers who lack mouse model expertise or capability, enabling them to have access to our mouse models without requiring them to generate or duplicate our models. This has the additional benefit of reducing experimental mouse numbers nationwide.

Medium-term: As part of the proposed work, we will test new cancer treatments in mouse models. Successful treatments in mice may lead to testing of the same drugs in human cancer patients.

Unsuccessful treatments in mice will spare patients from being treated with drugs that have little chance of working.

Long-term: Ultimately our goal is to improve survival and quality of life during survival of patients with lung cancer or mesothelioma.

How will you look to maximise the outputs of this work?

- Through collaboration with other scientists
- Through early dissemination of work in progress ahead of publication - this is achieved through presentations at national and international scientific conferences and through manuscript publication ahead of peer-review using pre-print platforms
- Through publication in well-regarded scientific journals
- Through sharing of new resources, datasets and new mouse models, the latter often well-before publication
- Through press release and social media announcements of major discoveries

Species and numbers of animals expected to be used

- Mice: 28,000 Genetically Altered 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.

Genetically altered (GA) mice present the most versatile mammalian setting in which to perform these studies. The vast majority of human genes implicated in cancer are highly conserved in mice, and there is an existing array of GA mice that allow for acute and long term manipulation of many genes implicated in cancer. These mice typically have a complete and fully functional immune system, enabling the investigation of tumour:immune interactions that may be tumour-promoting or tumour- preventing. Moreover, GA mouse models of cancer have proven to be much better predictors of human patient anti-cancer drug responses than any cell culture-based assay.

The vast majority of experiments will be performed in adult mice as both lung cancer and mesothelioma affect mature adult populations.

In some instances, mice will be aged until clinical signs require humane killing or to a maximum of 18 months if mildly symptomatic or asymptomatic.

Typically, what will be done to an animal used in your project?

Mice will be purpose-bred in order to combine multiple target genes of interest. These will typically be a mixture of cancer-causing mutations that, in most instances, are inactive until triggered under experimental control. Delivery of the activating "trigger" to specific target tissues or cell populations will be done in young adult mice, typically 8-12 weeks old. In our lung cancer models, this is usually accomplished by inhalation of inactivated, non-replicating, virus particles that carry the triggering protein. This is done in sedated mice. In our mesothelioma models, delivery of the "trigger" is accomplished by injecting similar viral particles into the chest cavity - taking precautions to avoid puncturing the lung. Additional drug-based triggers may be used alone or in combination with viral vector trigger delivery. Viral triggers are usually delivered once to any given mouse. Drug-based triggers may be given transiently, e.g. by injection or orally, or continuously, e.g. in feed/drinking water.

Almost all mice will subsequently develop tumours in the target tissue. These will vary from benign (ie. non invasive and non life-threatening) early stage cancer to more advanced stages of cancer, ranging from locally invasive to metastatic cancer (i.e. cancer that has spread to other parts of the body). Mice may be routinely monitored for tumour formation using non-invasive imaging technologies, such as ultrasound. Some mice will be humanely killed while their cancers are still at an early stage of development and these mice typically experience no clinical signs of cancer development - this enable us to investigate what is happening within the cancer at these early stages. Additional mice will be humanely killed as clinical signs emerge and progress, to investigate the biological processes involved in cancer progression. A limited number of mice will undergo more advanced imaging, e.g. by MRI or PET/CT, when more accurate or specific information about the cancer is needed.

A large subset of mice with cancer will be treated with established or potential anti-cancer drugs. Dosing concentration and treatment frequency will almost always be guided by previously published work. When this information is not publicly available, pilot studies on small numbers of mice will be used to establish effective well-tolerated doses. Duration of dosing will usually be limited to the minimum time required to determine if the agent has worked to improve health.

Mice will be continuously monitored by facility staff throughout their lifespan for general signs of ill health. In addition to general health monitoring, mice will be monitored by trained researchers with knowledge of the experimental circumstances, for clinical signs of cancer development/progression that are specific to each model. Monitoring will be routinely performed by visual inspection, which may be complemented by non-invasive imaging and/or regular body weight measurement. As clinical signs progress, so too will monitoring frequency.

Mice may be injected with agents that label key aspects of cancer cell biology, such as cell division, cell death, cell metabolism, areas of low oxygen or areas of reactive oxygen generation. This is usually done immediately before humane killing.

All experimental mice will be humanely killed at the end of each experiment.

Mice will thus typically undergo 5-6 procedures (e.g. tumour initiation; routine non-invasive monitoring; treatment with 1 or more anti-cancer agents; additional rounds of monitoring; injection with BrdU or equivalent; humane killing). Limited numbers of mice will undergo up to 5 procedures in addition to these steps (e.g. a second or third round of trigger administration; MRI or PET imaging on one or more occasion; irradiation; injection with reactive oxygen label in addition to BrdU immediately prior to humane killing).

What are the expected impacts and/or adverse effects for the animals during your project?

Mice in which either lung tumours or mesothelioma have progressed to advanced cancer commonly first show signs of breathing impairment or breathing alteration. Mildly impaired breathing without other clinical signs is usually followed by progressive breathing impairment (e.g. visibly rapid breathing and/or hiccups), reduced social activity or response to handling, and sometimes by weight loss. In our mesothelioma models, progression of clinical signs from mild to multiple moderate signs is usually quite rapid - occurring within 2-3 weeks. In our models of lung cancer, mildly impaired breathing may persist for several months before progressing to signs of more advanced cancer.

Treatment of mice with anti-cancer agents may be accompanied by signs of discomfort that vary by type of treatment. Commonly used chemotherapies almost always induce transient signs of moderate discomfort immediately following treatment, but these signs usually resolve within a couple of days.

Typical signs of discomfort include reduced movement, social withdrawal, or loss of appetite, which may be accompanied by rapid weight loss. These effects are usually short-lived and are lessened by provision of soft food.

Treatment with targeted agents, such as small molecule inhibitors, can be associated with side effects such as diarrhoea, pale feet, scruffy coat, scaly skin, weight loss or inactivity. These effects can often be lessened by reducing drug dose or dosing frequency, and typically reverse following cessation of treatment.

Repeated treatment with antibodies can sometimes provoke an acute whole-body allergic reaction that, left unattended, has the potential to cause sudden death. This reaction is very uncommon and typically specific to the antibody batch used.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The vast majority of mice on the breeding protocols will experience sub-threshold (>85%; Protocol 1) or mild (approx. 10% of total bred; Protocols 1 & 2) severity levels. Some mice (<5% of total bred) on Protocol 2 may experience moderate severity levels.

Most (>75%) mice on the GM Models of Cancer protocol (Protocol 3) will experience mild severity.

<25% may experience moderate severity levels.

50-75% of mice on Treatment of Cancer protocols (Protocols 4, 5, 6) may experience moderate severity with the remainder experiencing mild severity.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Cancer is increasingly recognised to affect and involve many more cell types than just those carrying mutations: cancer cells are supported by additional normal cell populations including fibroblasts, blood vessels and an array of immune cell populations. Indeed, the greatest therapeutic advance in recent years has been the emergence of immune checkpoint inhibitors that block the ability of cancer cells to hide from the immune system. Much of our proposed work now hinges upon further investigation of how cancer and immunity interact and influence each other. This can only be done in live mammals.

GA mice present the most versatile mammalian setting in which to perform these studies. The vast majority of human genes implicated in cancer are highly conserved at the molecular and functional level in mice, and there is an existing array of GA mice that allow for manipulation of these cancer- associated genes. Additionally, GA mouse models of cancer have proven to be much better predictors of human patient anti-cancer drug responses than any cell culture-based assay.

Which non-animal alternatives did you consider for use in this project?

We make extensive use of primary patient-derived tissue and fluid for investigating mesothelioma and available tissue microarrays for investigating lung cancer. I have considered the use of 2 and 3 dimensional cell culture and cell co-culture platforms as

alternatives to the use of live animals. I have also considered the use of lower organisms (e.g. flies, worms).

Why were they not suitable?

Patient-derived tissue and fluid samples allow for descriptive investigation but not mechanistic or functional studies. Additionally, both lung cancer and mesothelioma are diagnosed quite late (typically 30-40 years following exposure in the instance of mesothelioma), which limits investigation to advanced disease. To improve on early detection and early intervention, we need to understand what is happening in these cancers much earlier during their development, i.e. during pre-symptomatic stages of cancer development. This can only be achieved in living creatures.

Cell culture conditions are highly artificial and fail to fully replicate the conditions of a living organism. Moreover, while co-culture of some cell types together (for instance, cancer cells and fibroblasts) is now readily and routinely done, many cell types that are relevant for cancer are much more challenging to keep alive, and at best allow for only short-term (2-3 day) investigation, which is insufficient to determine the therapeutic benefit of anti-cancer treatments.

Lower organisms (e.g. flies) do allow for many aspects of cancer biology to be studied, however, modern drugs have been developed to specifically target the mammalian versions of the proteins that participate in cancer development, and often don't properly interact with the versions of these proteins that are present in lower organisms. Lower organisms also commonly fail to accurately reflect the unwanted side-effects of many cancer 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?

Estimated numbers of animals are based on recent (i.e. past 5 years) and current usage, grants funded to date through to 2028, and projected levels of staff for the foreseeable future. All of our models involve use of multiple GA genes because every human cancer involves multiple mutated genes. Interbreeding mice to generate the desired combinations of mutations required to accurately model human cancer unfortunately results in generation of large numbers of surplus mice that lack the desired genetic combinations.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

I have reduced the number of types of cancer that my lab investigates, from 4 major types of cancer to 2.

I have calculated total numbers of mice required to be bred and for experimental studies, and included full justification of those numbers and their associated costs, in each of my funded or currently submitted grant applications. Each funded application has been reviewed by external experts as well as by dedicated administrative staff, who carefully scrutinise mouse numbers to ensure they are appropriate and fully justified for the proposed work.

I routinely make use of the NC3Rs experimental design assistant, with estimates of variance and anticipated differences (e.g. between treatment cohorts) based on pilot studies or on previous cohorts of the same genetic model.

Where non-living experimental platforms are more appropriate for the scientific questions under investigation, my lab has and continues to make use of cell culture and co-culture to avoid unnecessary use of living 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 will continue to employ efficient breeding strategies to minimise the numbers of mice without useful genotypes arising within our breeding and maintenance colonies. This is achieved by sequentially enriching breeding stock for combinations of experimentally desired genetic alterations and periodically refreshing breeding stock to maintain optimal breeding health.

We will continue to use pilot studies when appropriate in order to estimate effect variability and effect size (e.g. for treatment with new anti-cancer agents) and to use the NC3Rs cohort calculation tool to ensure that appropriate mouse numbers are used in all of our studies.

We will additionally continue to collaborate with other researchers, e.g. those who lack mouse model capacity or expertise, thereby alleviating their needs to maintain large breeding stocks that would duplicate our own colonies.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 (GA) mice: GA mice are the most versatile mammalian setting in which to perform these studies. The vast majority of human genes implicated in cancer also exist in mice and serve the exact same purpose. There is a pre-existing array of GA mice already available that allow for short term or long term manipulation of many of these genes. Additionally, GA mouse models of cancer have proven to be much better predictors of human patient anti-cancer drug responses than any cell culture- based assay. In most (>85%) instances we will use mice that carry "conditional" alterations - that is modifications to a gene that require delivery of a protein or chemical "trigger" for the mutant version of the gene to be activated. This enables us to control the timing of mutation activation, and usually to limit mutations to a specific cell type or tissue (e.g. lung cells), minimising the potential for such mutations to affect other body parts.

Inflammatory particles: Asbestos, synthetic fibres with similar bio-physical properties to asbestos, and particulate matter under 2.5 micrometers in diameter (PM2.5) are known to provoke localised chronic inflammation of the lung and lining of the chest. Inclusion of these particles in our protocols is necessary to ensure that we capture this extremely relevant aspect of lung cancer and mesothelioma development. These particles are used at concentrations that alone provoke no immediate harm to the mice but over time do increase the risk of cancer development.

Non-invasive imaging: Tumour development can be monitored routinely using imaging technology such as ultrasound or the use of fluorescent proteins to label cancer or cancer-associated cell types. Use of these technologies is particularly helpful in mice that have yet to develop any clinical signs of cancer development, enabling the researcher to track the rate of early stage cancer progression without the animal experiencing any harm. More advanced non-invasive imaging technologies, such as MRI or CT, provide higher-quality images when these are needed, for instance to accurately measure tumour volume or to define areas for local treatment with radiotherapy. Mice are sedated during imaging and normally recover from sedation quickly without showing any signs of harm.

Treatment with anti-cancer agents: As is the case with human cancer patients, treatment with anti- cancer agents can have unwanted side effects. In many cases these are short-term responses to treatment and animals recover following cessation of treatment. Every effort is made to reduce the harmful impact of these treatments: Drug dose and frequency of treatment are usually guided by published information and both can be reduced if treatment is found to cause excessive suffering. Pilot studies will be conducted when any new drug or drug combination is tested, to ensure dosing is well- tolerated. Other approaches to minimise harmful impact include increased monitoring, provision of soft food and/or periodic injection with glucose/salt fluids to prevent dehydration.

Radiotherapy: Some mice will be treated with radiotherapy using specialised equipment that is purpose designed for use on small mammals. This equipment (the Small Animal Radiotherapy Research Platform - SARRP) delivers a narrow beam of irradiation, 7-8 millimeters in diameter, to a selected body region. Use of this equipment avoids irradiating vital organs or other unintended body parts, thereby minimising most of the unwanted side effects of radiotherapy.

Why can’t you use animals that are less sentient?

Many of the genes that are involved in human cancer are either absent in non-mammalian life forms, or are too different between mankind and non-mammalian species for anti-cancer drugs to work in the same way as they do in humans. Additionally, the immune system in non-mammalian species is often completely different to that in humans/mammals. Given the importance of the immune system in cancer development, and in response to cancer treatment, it is necessary to use a mammalian species such as mice in which the immune system is adequately similar to humans.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All staff receive specialist training on each of the GA mouse models they will work on. This is overseen by the NCTO and is led by the most experienced user of each GA model.

My team includes a clinically-trained ultrasound specialist to improve our use of this technology for monitoring of early stages of cancer development, particularly in asymptomatic mice.

We use a staggered approach to increase monitoring of mice from first onset of clinical signs, so that monitoring frequency increases as clinical signs progress, i.e. from mild to moderate clinical signs.

Pilot studies are performed on all new treatment/treatment combinations to ensure tolerability of dosing. If harmful side-effects are observed, drug concentrations and/or frequency of treatment will be reduced and a new pilot study started.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The NC3Rs ARRIVE guidelines: https://arriveguidelines.org/resources

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I routinely refer to the NC3Rs website and additionally receive regular emails on developments with NC3Rs guidance on best practice.

We have routine mouse user meetings (every 2-3 months) and AWERB events at the establishment to discuss adherence to best practice, any changes to guidance on best practice, any refinements that have been encountered in the literature, by word of mouth, e.g. at conferences, or indeed in-house.

Along with my own attendance, all members of my team are expected to attend these meetings.

We also refer to the MRC National Mouse Genetics Network, whose charter includes refinement and standardisation of best practice with dissemination across the entire network.

81. Tracking Atlantic salmon smolts to assess potential impacts of Marine Developments

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

Atlantic salmon, Acoustic tags, Marine distribution, Marine abstraction, Marine renewables

Animal types	Life stages
Salmon (Salmo salar)	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 better understand Atlantic salmon migration pathways in the Bristol Channel and provide data on the potential effect of coastal developments

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Fish which migrate into freshwater to spawn, such as Atlantic salmon (Salmo salar L.) may have to migrate past coastal developments to complete their life cycle. Developments such as tidal lagoons or major abstractions have the potential to impact on survival of both juvenile and adult stages.

The lack of data on migration patterns of these species, and hence potential impact, has impaired the ability of developers to assess impacts and propose suitable mitigation, compromising marine licence applications and potentially putting the fish populations at risk.

This project will provide specific data for an important coastal development area, and will look to quantify exposure of Atlantic Salmon smolts to a planned major marine abstraction and potential tidal range developments, as well as contributing to the development of a wider understanding of migration pathways in the Bristol Channel.

What outputs do you think you will see at the end of this project?

1. Evidence quantifying exposure of Atlantic salmon smolts from the River Wye and Severn to a major marine abstraction.

2. Evidence quantifying exposure of Atlantic salmon smolts from the River Wye and Severn to potential tidal range schemes.

3. Qualitative data on coastal distribution and migration paths in a wider area.

Interim reports will be provided to developers and environmental regulators. A final project report will be published and made publicly available. The more important results will be published in peer reviewed journals.

Who or what will benefit from these outputs, and how?

Lack of data on key marine species, including migratory fish, is recognised as a strategic information gap by both regulators and industry for assessing potential impacts of marine developments such as marine renewables (see the ORJIP report, 2017). Data for other anadromous species (twaite shad, Alosa fallax) has demonstrated significant potential risk to multiple populations of twaite shad from a new major abstraction (HInkley point C) and data are needed to understand whether similar risks exist for Atlantic salmon migrating through the area.

Developers and regulators will be provided with valuable data as the study progresses; migration data and survival will be analysed and reported regularly and disseminated through conference presentations and publications. Information specific to the area will benefit local regulation and inform evaluation of current and future development proposals. 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 (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 in excess of £0.5m to date) to help support the work.

Local angling associations are keen that evidence is developed to ensure that both the fish population and fishery are protected through the regulatory process for marine developments. They have provided/are providing practical assistance with our fieldwork, including fish capture.

EDF Energy Ltd, the developer of Hinkley point C, are also providing access and assistance and both they and environmental regulators will be provided with relevant evidence to assess the potential impact of their abstraction.

How will you look to maximise the outputs of this work?

Collaborating across multiple organisations and river systems will maximise data value and the potential for novel outcomes.

We are already working closely and collaborating with Natural Resources Wales, Natural England, Environment Agency, and the Devon and Severn IFCA, EDF Energy Ltd, as well as local angling interests and organisations such as Marine Energy Wales and the Game and Wildlife Conservancy Trust.

We expect to produce reports, conference papers and peer reviewed publications.

Species and numbers of animals expected to be used

- Salmon (Salmo salar): 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.

At the current time Atlantic salmon and sea trout populations in many parts of the UK are at historically low levels. Atlantic salmon migrate to sea as smolts. During these migrations they may be killed or their migration success compromised by developments such as abstractions or marine turbines associated with renewables.

Atlantic salmon smolts leaving the rivers Severn, Wye and Usk may be at particular risk during their migrations from a new marine abstraction (the largest in the UK), scheduled to begin operation in 2027, and from the potential development of tidal lagoons for energy generation in the Bristol Channel.

This study aims to look at Atlantic salmon migration paths, and will focus on quantifying the potential exposure of smolts to the developments described above.

This is essential information for regulatory discussions and for managing and protecting salmon populations.

Typically, what will be done to an animal used in your project?

Fish will be captured using rod and line, or nets and traps specifically designed to avoid damage. 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.

Fish will then be transferred to a well aerated recovery tank and monitored for normal behaviour (holding their position and actively swimming).

Once the fish are recovered from anaesthesia they will 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 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 fish are only released once they are behaving in a normal manner within the release tank, when they are able to swim upstream against the flow.

Expected severity categories and the 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 all fish tagged. 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 are to look at the behaviour and distribution of Atlantic salmon (Salmo salar L.) in the wild in order to gain information to manage and protect the species in the context 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?

Theoretical modelling and expert opinion has already been utilised to look at potential distribution and movement in the study areas. Comparison of predictions using these methods and actual data already collected on twaite shad has demonstrated that, in the absence of real data, expert opinion and modelling approaches may produce highly misleading and inaccurate results.

Specific empirical data for salmon smolt migration to validate opinions and models are therefore essential to underpin environmental impact assessments and regulatory decision making.

Why were they not suitable?

See above. In the absence of migration studies and actual movement data for Atlantic salmon and sea trout in the Bristol Channel there is no basis to validate these models and their predictions. Data from similar studies of twaite shad has demonstrated that expert predictions in the absence of real data were very wrong.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We need to use sufficient fish to provide quantifiable estimates of detection rates in each of the development areas, which can be compared with the number of tagged fish successfully emigrating from each river, to enable calculation of the proportion of the population exposed to risk. The actual numbers required will be determined by emigration success, and by the proportion of tagged fish entering the development areas, which are currently unknown. Proposed sample sizes (up to 150 on each river) have been informed by results from emigration success and detection rates from our other studies of Atlantic Salmon (river Tawe) and twaite shad (rivers Severn, Wye and Tywi).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Proposed sample sizes (up to 150 on each river) have been informed by results from emigration success and detection rates from our other studies of Atlantic Salmon smolts (river Tawe) and twaite shad (rivers Severn, Wye and Tywi). The study will be undertaken on a 'pilot' basis, with up to 75 tagged in each river in year 1, data reviewed, and further tagging undertaken in a second year if necessary.

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, with the tags specifically designed for the purpose, and the smallest that will provide the necessary output and battery life required for the study. The methods we are using 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 understand observe behaviour and distribution of Atlantic salmon smolts under natural conditions, including 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?

Capture method

Depending on site availability we will use one or a combination of trapping (rotary screw traps (RST), wolf grids or similar), fyke nets modified to minimise damage, or rod and line. All of these methods have been demonstrated to provide fish in good condition for tagging.

Choice of tags

We are using the smallest tags available consistent with the objectives of the project, including tag life and acoustic output needed for tracking in the marine environment.The tags we are using are specifically designed by the supplier 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 technique we are using ensures water circulation across the gills throughout the procedure.

When tagging during dark hours light will be kept to a minimum to reduce stress. Aseptic surgery techniques and single use scalpel blades and suture needles will minimise risk of infections.

Each incision will be covered with a suitable temporary wound barrier to provide a waterproofing barrier to protect the wound in the initial stages of healing. Sutures will be checked prior to transfer into recovery and holding tanks.

Smolts will be monitored and only be released in groups when exhibiting normal swimming behaviours.

All procedures will only be performed by suitable trained and qualified individuals (ie PIL holder; training and competency record kept by NTCO)

Smolts will be released in groups to maintain shoaling behaviour. 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 have taken best practice advice from various partners and Universities, all of whom 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 continue to attend conferences, such as recent SAMARCH, Unlocking the Severn and IFM workshops, which bring together salmonid tracking researchers. As well as learning ourselves, we will present data at these

events to share it with others. We will 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

82. Neuroimmunity in Obesity

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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, neuroimmunity, therapy, brown-fat, thermogenesis

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?

Obesity and its contribution to chronic illness is an unmet medical need. Our project aims to understand the interaction between the immune system and the nervous system at nerve fat junctions and how it controls physiology, fat mass, associated increases in blood pressure, and metabolism.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 important due to the potential benefits that could be derived from our research for society and public health.

Nearly one third of adults in the UK is obese and yet no safe medications are available to patients to alleviate this disease.

The health burden of obesity is overwhelming: diabetes, high blood pressure (hypertension), heart disease, stroke, nerve damage, kidney disease, blindness, limb amputation, among others — all contribute to the reduced quality of life and reduced life expectancy experienced by people living with this disease. In addition, the financial burden on healthcare system is significant: £10billion each year (10% of the annual NHS budget) is spent only managing diabetes (www.diabetes.org.uk), let alone the financial burden of all other diseases associated with obesity. Finally, the loss of productivity in the workforce due to ill health or social stigma of obese patients is substantial. As the global population is facing an obesity and associated diseases epidemic, research into this area is a medical, economic and ethical priority. We hope our work will pave the way to the development of a new generation of anti-obesity medications, and therapeutic strategy, through the identification of novel targets that control fat mass.

What outputs do you think you will see at the end of this project?

- New information regarding the junction between fat and nerves.
- Establish how immune cells interact where fat and nerves meet.
- Information of the neuro-anatomical map of the sympathetic (fight or flight process) nervous system.
- Decipher how the sympathetic nerves impact blood pressure and whole-body heat regulation.
- Future treatments for obesity that would be independent of food intake
- Our outputs will lead to peer reviewed scientific publications in recognised medical journals.

Who or what will benefit from these outputs, and how?

In the short-term our publications are academic in nature, so we would provide basic scientific discoveries to obesity research by uncovering new knowledge. Our laboratory has a record of publishing in high impact publications and we would hope to continue in kind.

In the long term our work could benefit patients and clinicians as these discoveries could form the basis for a therapy influencing these mechanisms to control whole body weight, independent of food intake which would be of benefit to society and public health at large.

How will you look to maximise the outputs of this work?

We will maximize the outputs of this work through collaboration. We regularly donate breeding pairs of transgenic mice to other research groups within our establishment, as well nationally and internationally.

Our lab has a track record of disseminating knowledge through high impact publications and we hope to continue that along with presenting our research at academic conferences.

Species and numbers of animals expected to be used

- Mice: 57000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 studies of whole body metabolic homeostasis to be relevant to humans, it needs to be conducted on mammals. It is essential that our research project is carried out using mice, as there is simply no alternative for the genetically altered techniques we utilize. Adipose (fat) homeostasis, food intake, hormone secretion, action, stability, and clearance are regulated by a myriad of circulating factors (each with their own pattern of systemic regulation) and the contributions of multiple organ systems, which cannot possibly be recreated in vitro therefore, these processes must be investigated in a living mammal to ensure our results are physiologically relevant.

Mice are international gold standard for the transgenic manipulation of body weight homeostasis our lab engages in. We use juveniles and adult life stages to prevent any age associated long-term, adverse effects caused by these genetic changes.

Typically, what will be done to an animal used in your project?

Genetically altered mice will be bred using natural methods. The mutations that these mice carry are not expected to have any harmful phenotypes which exceed a mild severity. Our obese model is a well-established mouse strain, which although larger in size will experience low grade diabetes which does not affect its normal wellbeing. Some of our mouse strains will need to be given a substance, typically tamoxifen, to activate or deactivate the gene of interest.

Mice will be exposed to a several different challenges to assess weight loss or gain. These challenges include being fed a diet with altered nutrient content such as high fat, undergo fasting for up to 18 hours on up to 4 occasions, exposure to a fluctuation in temperature compared to the normal conditions that they are housed in. These are low temperatures between 4 and 10oC for a period of 24 hours and then up to 35oC temperatures for up to 10 weeks. Mice tolerate the heat well, being desert animals in origin.

Other challenges will involve the administration of substances. These substances will be from groups that are gene inducers, metabolites that alter a feature of the metabolism or allow us to observe a particular metabolic process, substances that allow us to study the role of the metabolism in obesity and therapeutic substances.

During these challenges mice may have a blood sample taken to allow us to assess the metabolic process. Also, they may have their blood pressure taken using a cuff applied to the tail, which mimics the arm cuff applied to humans. This is non-invasive and causes minimal stress to the animal.

Other tests will include taking core temperature readings via a rectal probe, taking external temperature readings via a thermal imaging camera, activity monitoring using infrared beams, motion sensors and videos. As these readings are performed in free moving animals in their home cage it is not stressful for them. Another useful test will be a test to measure the uptake of glucose or insulin.

This test involves administering a bolus of either glucose or insulin and at time points over two hours taking a very small blood sample to see the level of these in the blood stream.

Activity plays a large role in obesity and some animals in this project will be subjected to behavioural tests of motor co-ordination and function on up to 20 occasions. The animal may be required to perform up to 2 tests per day.

Some mice will be given substances that label cells or that will trace a metabolic pathway.

Some animals will be kept awake during their inactive period, which is our daylight hours, by applying a mild physical stimulus or by presenting them with novel objects which will cause them to investigate rather than sleep.

To investigate the role of the immune system in metabolic processes some animals will be exposed to a dose of irradiation that will deplete its bone marrow and then the immune system will be regenerated 24 hours later by the administration of cells. These mice will then undergo some of the challenges described earlier.

Typically, we use standard administration routes however if the dosing regimen is over a longer period of time we may surgically implant a device that will deliver the substances without the need to perform injections on a daily basis.

What are the expected impacts and/or adverse effects for the animals during your project?

We have listed few expected adverse effects in our substance administrations because the effects are highly transient nature. Similar can be said for our environmental challenges. For example, in a cold challenge we chill the mice to a degree to where they increase thermogenesis but they must not shiver. Our fasting protocol has mouse weight loss as high as 15% to elicit the changes in body weight homeostasis necessary to study neuro-immuno-metabolic mechanisms that fasting induces in the context of health and obesity.

The tests listed have been refined over many years to cause the least disturbance to the mouse possible, whilst gaining suitably robust data to answer our research questions. It is important to remember that stress or pain will impact metabolism and subsequently confound our experimental data sets: therefore, there is a strong scientific as well as ethical rationale for us to avoid inducing stress or pain. Furthermore, our breeding strategy and mouse lines exhibit mild (if any) 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)?

Mice: 24% subthreshold, 65% mild, 11% moderate

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

It is essential that our research project is carried out using animals, as we require multiple organ systems to interact. Adipose (fat) homeostasis, food intake, hormone secretion, action, stability, and clearance are regulated by a myriad of circulating factors (each with their own pattern of systemic regulation) and the contributions of multiple organ systems, which cannot possibly be recreated in vitro therefore, these processes must be investigated in a living mammal to ensure our results are physiologically relevant.

Which non-animal alternatives did you consider for use in this project?

We considered in-vitro and in-silico approaches and also organoids.

Why were they not suitable?

We use in-silico bioinformatic single cell approaches as clues to guide our research and reduce the number of pilot experiments and therefore reduce mouse numbers.

The organoids are not suitable because to date, there is no good sympathetic neuron organoid system available that can reproduce the entire physiological repertoire of cell types, the level of maturation and function of the sympathetic nervous system.

We will always replace in vivo experiments with in vitro experiments where possible, such as testing expression or basic function of a gene/protein or optimising drug dosage. This data will then inform the subsequent in vivo testing which will establish physiological relevance.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated our numbers by using typical variation from our previous experiments. This has given us a calculation of the minimum number of animals to be used whilst ensuring that the results are statistically significant. We are also factoring in an increase in personnel within the group. Typically, we use groups of 5-10 animals in our studies. We have used our annual return of procedure 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?

We reduce the number of mice use by maximizing the quality of our experimental design using the experimental design assistant published on the "National Centre for the Replacement Refinement & Reduction of Animals in Research" website. Ensuring the statistical power of the results outputted.

Mice will be housed in identical conditions, and experimental animals will be compared with littermate controls to minimize variability. For this reason, we will also ensure that procedures (for instance, evaluation of hormones in blood) are performed at the same time of day. Male and female mice will be used in equal numbers (approximately), unless phenotypes are sexually dimorphic. We will rely on our extensive experience to ensure that experiments are carried out with the highest technical precision to avoid wasting mice and when possible all organs will be harvested from mice to maximise the data output from

each animal. Technical accuracy will reduce noise in the data, meaning that fewer mice are required to achieve sufficient statistical power to test a given hypothesis. Where possible, experimental outcomes will be collected with the observer blind to genotype/treatment to reduce bias, and unblinding will only take place following data analysis. Any animals excluded from data analysis will be reported.

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 strategies 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.

We will use genetically modified laboratory mice. Genetic alteration has played a key role in dissecting the biological basis of human obesity and is only available in the mouse or to a lesser extent in the rat. A prime example of this is the identification of the hormone Leptin, first discovered in mutant obese mice, and one year later in humans, that regulates body weight. Therefore, the mouse is the lowest vertebrate that can be used to achieve the purpose of this project with enough aspects of genetics, anatomy and physiology shared with humans to generate biologically relevant data that can ultimately be used to develop new therapies for obesity and associated co-morbidities. They may be transgenic from conventional breeding strategies and genotyping. The transgenic lines utilized may be obesity models or conditional or constitutive knockout models. For instance, the leptin-deficient ob/ob mice, as well as target gene-specific diptheria toxin receptor or CRE/Lox reporter mice that are bonafide reporters of our cells of interest. As our lab focuses on the sympathetic-immune cross talk that regulates obesity, we use the least intense obese models (ob/ob and diet-induced obesity) rather than classical diabetes models that exhibit much more severe phenotypes.

We have chosen to utilize the simplest and efficient experimental methods that avoid any unnecessary pain and animal suffering. We will administer substances using the least painful route that preserves the intended effect of the compound, for instance Clenbuterol

in drinking water instead of injecting it as the drug is sufficiently stable. None of the substances that we have proposed to administer have long- lasting adverse effects that extend beyond the actual administration method. Furthermore, none of the techniques that we propose, such as fasting, cold shock or intraperitoneal injections should result in long-lasting effects that will persist beyond the actual time of the procedure. If deemed necessary, proper analgesia will always be provided to ensure that pain and suffering is minimized to the best of our abilities.

Substance administration in terms of volumes and frequencies will follow s the published best practice.

Intrathecal injections under general anaesthesia delivered using a thin micro needle syringe guided by a stereo-taxic apparatus) ensure minimum invasiveness for delivery of substances to the sympathetic nervous system. Adverse effects should cause no lasting harm and consist of no more than mild, transient post-surgery distress. These delivery methods prevent unnecessary invasive surgical procedures that would achieve the same results but with increased complications e.g. wound dehiscence.

Intra-adrenal injections ensure minimal invasiveness for delivery of substances of certain directly to the adrenal glands, this will prevent the effects of systemic delivery. For instance by locally delivering diphtheria toxin on the adrenal glands of a mouse expressing diphtheria toxin receptors in all myeloid cells, we only target those residing in the adrenal glands and not other essential tissues such as microglia. Adverse effects should cause no lasting harm and consist of no more than mild, transient post-surgery distress. In contrast, systemic effects that result in depletion of microglia are much more severe, mimicking symptoms of multiple sclerosis, which we avoid with local injections.

Where repeated drug delivery will be required over a course of days or weeks, surgical implantation of minipumps will be performed in favour of repeated injections. Mini-pump implantation is a quick surgical procedure that should cause no long-term effects to the movement or well-being of the mice and reduces the stress of repeated handling and injections. It is therefore a preferable refinement of repeated substance administration.

Blood pressure and heart rate measurements can be taken by the tail cuffs, as you do in humans with an arm cuff. This method, widely used in the field and relatively cheap to implement, provides non- invasive measurements of blood pressure.

If only activity monitoring is required then it will be done using the least invasive method, namely using home cage monitoring in individually ventilated digital cages for up to 4 weeks

Why can’t you use animals that are less sentient?

We're unable to use less sentient animals such as fish or fruit flies because they are not an accurate model of adult human body weight homeostasis or the therein. One cannot model adult obesity on immature or terminally anesthetized animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

To help reduce any stress or suffering that animals may be subjected to, mice are monitored carefully for any signs of adverse effects. Clear humane end points are set out in each protocol as well as intervention criteria. Phenotypic profiles including humane endpoint guidelines for strains with known adverse effects will be clearly displayed where possible. If a mouse appears to be unwell or deviates from the phenotype of a normal healthy mouse, but does not reach its humane endpoint the vet may be consulted. If necessary, appropriate treatment to relieve pain or distress will be given following advice from the vet. If no improvement is observed within 24 hours of intervention, the animal will be humanly killed.

Dose levels for substances will be kept to a level that is not expected to cause any clinical symptoms but still enables us to achieve our scientific aim.

During such surgical procedures, appropriate anaesthesia will be used in line with best surgical practice.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will consult the National Centre for the replacement, refinement and reduction of animals in research for best practice guidance on the 3R's. We will also follow guidance from the Laboratory Animal Science Association.

We will also work to the ARRIVE and PREPARE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

By staying in close contact and discussion with veterinary and technical staff at our animal facility who are experts in this advancing field. We will sign up to the NC3R's newsletter, attendance at welfare meetings held within the establishment.

83. Optimising Islet Transplantation in Type 1 Diabetes

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- 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

diabetes, engraftment, islets, insulin producing cells, insulin

Animal types	Life stages
Mice	pregnant, adult, juvenile, neonate, embryo
Rats	pregnant, adult, juvenile, neonate, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Transplantation of insulin producing cells into the liver of people living with Type 1 diabetes is a procedure that can stabilise blood glucose levels. However most transplanted cells die and the procedure rarely results in freedom from insulin injections and the production of insulin from the transplanted cells wanes over time. Our research is focused on improving the survival and function of these insulin producing cells in the liver by applying different therapies in the liver. The liver is used as the site for an islet transplant as it mimics the human procedure where type 1 diabetic patients receive islets into the liver as opposed to other sites. This is because islets can be easily introduced into the liver through the hepatic portal vein by non-invasive imaging. This particular approach is not categorized as

major surgery, leading to swift patient recovery. Considering these benefits, animal studies consistently opt for the liver as the preferred organ for transplantation 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?

If we can improve the number of insulin producing cells that survive after transplant then we improve the function of these transplanted cells not only in the short term but also in the longer term. This would mean that more people would achieve freedom from insulin injections, the quality-of-life would improve and there would be decreased complications from their diabetes with cost savings.

By doing this animal work we can test what therapies alongside insulin producing cells would lead to these cells surviving longer and this would result in all the benefits listed above this work is important to do before performing first in human studies. Unfortunately there is no real alternative other than transplantation into mice that can be used to test these therapies.

What outputs do you think you will see at the end of this project?

Sharing our research findings through published papers and presentations at conferences will make a positive impact on the scientific community within the next five years of this project. By consistently publishing new information in this field, we will contribute to the advancement of knowledge.

Additionally, participating in conferences will give us the opportunity to share our discoveries, engage with other researchers, and foster collaborations. These interactions will help disseminate our work, spark valuable discussions, and collectively drive progress in the field.

Who or what will benefit from these outputs, and how?

In the long run, the outcomes of this project could significantly benefit individuals with diabetes. People living with diabetes, along with their families, often experience a lower quality of life due to the inconveniences and unpleasantness associated with daily insulin injections and dietary restrictions.

Unfortunately, current therapeutic options for diabetes are limited, primarily relying on insulin therapy. Some patients eventually require the transplantation of donor islets to regain control of their blood sugar levels. However, these transplants have a limited success rate and lifespan, often necessitating repeated procedures.

Therefore, discovering new approaches such as innovative medications, cell therapies, or immunotherapies to enhance the success of transplanted islets/insulin producing cells and prolong their effectiveness would be a significant breakthrough in diabetes treatment. Throughout this research, we will investigate how transplanted islets and similar insulin-producing cells attach and function, aiming to identify factors that optimize their integration and explore the underlying mechanisms. If our successes in preclinical models can be translated to humans, it holds the potential to make a profound impact.

Moreover, the improved durability and effectiveness of islet transplants could lead to healthier lives for patients over an extended period. This would allow them to remain productive and engaged in their work for a longer time, while also reducing the burden of care. Ultimately, our work seeks to offer new avenues for enhancing the lives of those affected by diabetes and potentially revolutionize their treatment options.

How will you look to maximise the outputs of this work?

In order to maximise the outputs of this work we will seek to publish the results of all studies conducted under this project and where possible open access journals to ensure the widest dissemination of our research. For all publications we will use our social media accounts to share the data with the field and patient groups/charities.

Where possible we will seek to ensure we release accessible statements about our results specifically for the diabetes patient community and their families.

Species and numbers of animals expected to be used

- Mice: 4500
- Rats: 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.

We are focusing our work on diabetic adult mice as this best reflects the human/population that we are treating. In some cases where we are testing out human cells we will use very specialised mice that lacking immune system that will not reject these cells.

Typically, what will be done to an animal used in your project?

Typically we will induce diabetes in these mice with a chemical. We will then transplant these mice with insulin producing cells into the liver via the main vein going to the liver. In order to do this the mouse has to be under anaesthetic and the abdomen opened to access the vein to the liver. The transplantation of insulin producing cells will be accompanied by a therapy alongside it. We will then survey the mice over a period of up to

16 weeks after their transplant to see what effect the therapy has had and whether we have achieved a greater cure rate of the diabetes in the mice that received these therapies.

What are the expected impacts and/or adverse effects for the animals during your project?

The typical impact is of weight loss due to the induction of diabetes as well as excessive passing of urine if the diabetes is not controlled by insulin or a therapy that helps control the blood glucose levels. The duration of this can be short-lived up to 3 days; any excessive weight loss would be treated appropriately and if there was an issue with respect to this then we would discuss with the vets and deal with this situation as per agreed protocols.

As the animals undergo surgery another adverse event is blood loss and other complications related to the anaesthetic. Intraoperative blood loss can occur at the time of injection into a main vein in the liver and is controlled by haemostatic gauze. In less than 5%, mice do not respond to intervention and are culled before recovering from anaesthesia. Post-operative blood loss is very rarely observed (>0.1%).

Animals will experience surgical procedures including having transplants placed under the outer layer of the kidney or into the liver. If their diabetes is cured, they may also have a kidney with the transplant in, later removed to demonstrate that the diabetes then returns: this indicates that the removed kidney contained the cells that cured the diabetes. They will have imaging scans that need dye injected into their veins in order to make the body parts clearer.

Surgical techniques can be uncomfortable and potentially painful and are helped with pain relief including anti-inflammatory medications.

Expected severity categories and the 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 following are moderate severities:

Although some weight loss is seen after the transplant, we expect less than 5% to suffer from weight loss of >15%;

Complications from anaesthesia <1%

Intra-operative bleeding during HPV injections <5% Post-operative blood loss <0.1%

Animals may develop insulin dependent diabetes that requires daily injections of insulin to be administered (~80%)

Animals can experience episodes of low blood glucose (hypoglycaemia) that require injections of glucose to restore normal blood glucose levels (<3%).

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Due to the complexity of diabetic disease and the involvement of multiple cell types it is not possible to fully mimic diabetes and the therapies and techniques proposed here fully by non-animal alternatives.

Which non-animal alternatives did you consider for use in this project?

Where possible before using animals we utilise several cell culture models that we have generated which can mimic parts of the process, for example drug effects and cell interactions with insulin producing cells studied in a dish (in vitro studies). With these in vitro studies we can study these cell and drug/cell interactions and evaluate any potential issues prior to carrying out studies in animals.

Studies in animals are important to perform as we need to evaluate islet function in the context of an immune system which cannot be modelled in a dish. Also we need to evaluate the function of transplanted islets after they have formed blood vessels with the transplant site (in the liver) and the islets take time to engraft and again this cannot be observed in a dish. We are always evaluating new methods to study the interactions between islets and the transplant site but at the moment examining islets with organoids is not a viable alternative. We will continue to refine and reduce our use of animal models as ex vivo models progress.

Positive approaches and results seen in non-animal models will be taken forward into in-vivo studies but we may need to take other approaches that cannot be adequately tested in vitro forward into animal models.

Why were they not suitable?

The in vitro models we have give us vital information about the therapies and how they interact with insulin-producing cells in a dish but this is only part of the interaction and is only a snapshot of what we want to study. In a dish, insulin producing cells do not have the interactions with other cells nor do insulin producing cells survive for longer than 4-5 days outside of the body in these dishes.

Since we wish to study how insulin producing cells which are infused into the liver, interact with the liver and how blood vessels form between these insulin producing cells and the liver - which takes place over a period of four weeks it is necessary to use an animal model. If we can improve the rate at which blood vessels form between the insulin producing cells and the liver then the chances of an insulin producing cell surviving for longer is much increased as we have already shown in our work.

This animal model gives us a chance to apply different therapies to the liver or islets and study for example blood vessel formation as well as insulin production from the cells. The way in which the animal responds to these therapies eg. its immune response is also an important factor and therefore these animals are critical in the assessment of all these parameters.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers have been calculated from previous PPL and from the planned experiments we have over the next 5 years.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have used the NC3R's Experimental Design Assistant; wherever relevant we will use the results from in vitro work to inform the in vivo work.

For example in the experiments with cell and other therapies we will look at the cell therapy in combination with insulin producing cells/islets in a dish and determine cells and therapies with the greatest impact on the insulin producing cells before commencing work in rodents.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will utilise small pilot studies with for example dose finding to optimise the number of animals in the project. We will also use the pilot studies to the full extent and examine for example blood levels of key signals that are likely to be important in conjunction with an examination of the tissue that the insulin producing cells are implanted into as well as an examination of other tissues to better inform later studies.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We propose to use mice and rats in order to achieve the objectives of this proposal. Rats and mice have been proposed to be used in this study as the majority of published data is available from these species.

For certain studies rats are required to be used rather than mice for example to detail imaging which can be limited in the mouse due to their small size and thus only in rat can we gain meaningful measurements.

Mice will be used as they can be transplanted via the hepatic portal vein and end points determined easily with relatively small numbers of islets. It can also important to demonstrate that results found in once species are transferable to another and not specific to the species used. This is particularly important when looking for translational strategies. Furthermore, data obtained from these models in mice and rats have been demonstrated to be relevant to the human condition of islet transplantation. In order to study islet transplantation in type 1 diabetes an animal model has to be used as both processes involve several cell types and an inflammatory response which we are unable to replicate in cell culture.

For these animals the experiments are carefully planned to use the lowest number of animals to have a statistically valid group number. Control mice will only be used when essential and where possible, historical tissue will be used. The length of time the animals are on these protocols is carefully selected so that animal suffering/distress is as low as possible.

The inclusion of PET imaging should in future allow us to refine our ability to assess the fate of islets.

For substance administration we will use the most appropriate route which causes the least animal distress/suffering. For example using where possible osmotic minipumps to avoid repeated administration of substances.

Why can’t you use animals that are less sentient?

Islet transplantation in people with Type 1 diabetes is a procedure that may be appropriate for people in middle age and later life so using immature life stages would poorly model the mechanisms we seek to understand. Data obtained from mice and rats have been demonstrated to be relevant to the human condition which has not been shown fully for species which are less sentient. We will refine our studies and decrease animal distress/suffering.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have refined the models we use over previous PPL projects. We now routinely group house post- surgery which has improved animal post-surgery condition scores. We have increased analgesia post surgery up to 72hrs when required which has also improved animal condition scores. We use animal monitoring sheets for all animals who have had diabetes induced or islets transplanted, regardless if asymptomatic or not. These sheets document daily weight, daily glucose levels, volume of daily insulin given if required, observations of condition of animals. This ensures that all animals are carefully monitored and ensures we maintain humane endpoints for all animals used.

For certain procedures for example blood glucose monitoring we train the animals so they are used to the procedure prior to taking measurements. This allows the animals to get used to the handling and measuring apparatus reducing distress when measurements are made.

We closely monitor the cages and check them twice daily. This includes changing any damp bedding, replacing water bottles, scattering food cubes on the floor. We also provide an enhanced diet to any animal losing weight, including sunflower hearts and baby food/mashed rodent food.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Where possible we used published best practice guidance in our experiments. For example for our diabetes models we used the guidance of the Diabetic Complications Consortium (DiaComp) publications. For the other models and procedures we have written SOPs which we use for training purposes and to ensure best practice.

We also follow the LASA guidelines for substance administration and standards for aseptic surgery.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

To ensure that we stay informed about advances in 3Rs we will attend our University 3R's day held annually and the PIL refreshers course which updates on important new

advances for example tube handling. These new advances will then be utilised during this project. We will also make use of the NC3Rs website news to ensure we are up to date.

84. Understanding the role of systemic inflammation in cardiovascular disease and obesity

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

Cardiovascular, Obesity, High blood pressure, Diabetes, Therapy

Animal types	Life stages
Mice	juvenile, adult, 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?

This project aims to understand how increased fat in obesity causes cardiovascular diseases such as high blood pressure and diabetes, and develop new ways to treat 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?

Cardiovascular disease is the most common cause of death in the world, and one of the most common causes of disability. Obesity is very common, and it is one of the biggest

causes of cardiovascular disease. However it is not easy to lose weight by dieting, and most people will put back on the weight they lose on a diet within two years. Treatments for the diseases caused by obesity are not very effective, and many of them have unpleasant side effects which leads to people not taking their medicine. Therefore, we need to understand how obesity causes disease, so that we can develop better treatments.

What outputs do you think you will see at the end of this project?

These studies will generate new data to advance scientific knowledge of the development of cardiovascular diseases in obesity. These data which will be published and presented to the cardiovascular science community. This knowledge may identify new approaches to treatment of obesity-related diseases, and new drug targets. In addition, these data may refine animal models used by others.

Who or what will benefit from these outputs, and how?

These studies will further the knowledge of the research group, and our future research will build on these findings. Ultimately, our findings may highlight new targets from drug development for pharmaceutical companies or new approaches to treatment of obese patients by clinicians; thus translating into patient benefits.

How will you look to maximise the outputs of this work?

Data will be published in open-access journals and presented at scientific meetings. Where feasible, data and resources will be shared with other researchers and collaborators.

Species and numbers of animals expected to be used

- Mice: 2440

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 structure and function and the mouse cardiovascular system closely models that of a human. The majority of our experiments will be conducted on mice that are around 4 months old; when they are full grown. This is because we are interested in the effects of obesity, therefore many of our studies will involve using mice fed a modified diet to mimic obesity, and we need to allow time for mice to feed and grow. Most animals used under this licence will be purchased from appropriate suppliers, however for some experiments we will breed our own mice that have been genetically modified in a way that will help us

study diseases. Breeding will involve the use of neonates/embryos, adults, pregnant adults and juveniles.

Typically, what will be done to an animal used in your project?

The majority of mice in this project licence will undergo mild, non-invasive or minimally invasive tests on their cardiovascular systems very similar to those done in humans, for example blood pressure, recordings of electrical activity in the heart, or blood tests. These may involve gently restraining the animals, and may be done multiple times. For some experiments, like echocardiography, we will need to anaesthetise the animal to be able to take for these recordings.

To study how obesity causes diseases, mice may be fed a modified diet such as a high fat diet, usually for around 3 months. We will also investigate how exercise in obesity might treat diseases. For these experiments, some mice will be trained to exercise either on a treadmill or by swimming, usually for around 6 weeks.

For some experiments, we will administer by injection or oral gavage substances such as potential new treatment or drugs which mimic disease, so that we can study how they work. This is usually daily for a short period of time e.g. 5 days.

Some animals will be infected with a parasitic worm.

In a small number of mice (~7%), a device that can record blood pressure and/or recordings of electrical rhythm in the heart will be surgically implanted under the skin.

What are the expected impacts and/or adverse effects for the animals during your project?

For some experiments animals may need to be gently restrained or anaesthetised, which may cause mild stress but not for long and the animals will recover almost immediately.

For mice fed a modified diet, we will ensure that diets are palatable and all of their nutritional needs are met. Sometimes these diets may make their fur greasy, but this won't cause any lasting harm. These diets may cause the animal to become obese and have high blood pressure or diabetes. In our experience, the obesity has no effects on their ability to move around and they don't exhibit any symptoms of illness, so although they are fat they appear to be normal and will not suffer.

Some studies have suggested that exercise can cause stress in mice. In our experience, we have never seen any signs of stress in our models. Furthermore, we have measured stress hormones in their blood and found they are normal. Mice will not be exercised to exhaustion.

Animals receiving injections or having blood taken will experience mild, transient pain, but no lasting harm.

Some animals will be infected with a parasitic worm, these worms are not harmful and do not normally result in any suffering or distress.

In the small number of mice (~7%) undergoing surgery they will experience some discomfort for a few days after surgery and some mild to moderate pain. Also, they may lose weight in the first few days but they should make a full recovery.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

10% of mice will experience moderate severity.
59% of mice will experience mild severity.
31% of mice will experience sub-threshold severity.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We wish to study how obesity causes cardiovascular diseases. Cardiovascular diseases involve multiple organs that interact with each other. Specifically, we are interested in how fat causes these cardiovascular diseases, which is widespread around the body. Therefore, because of the complex and widespread nature of these diseases, to be able to study them we need an intact organism with a similar cardiovascular system to humans.

Which non-animal alternatives did you consider for use in this project?

We have considered and researched the literature on organ-on-a-chip technology, cell culture and computer models.

We cannot consider non-protected species such as fruit flies, as they do not have a comparable cardiovascular system to humans for study. Fruit flies may be useful in studying genetic causes of cardiovascular disease, however we are studying the effects of lifestyle (obesity) on cardiovascular disease, and this could not be replicated in a fruit fly.

Why were they not suitable?

There are no existing computer models of blood vessels that include modelling the effect of fat on the vessels, which is key to our studies. State of the art organ on a chip technologies cannot yet replicate the contraction and relaxation of blood vessels, which is important to studying how blood pressure becomes increased in obesity. The same applies to cell culture. None of these methods mimic the complex nature of interactions between multiple organ in the body. As we wish to study the onset and progression of disease in obesity, we need an intact, live system. Prior to performing studies in live animals, we use tissue taken from culled animals to conduct experiments outside of the body to direct our research. Specifically, we can dissect out arteries with their surrounding fat intact and measure their contraction and relaxation in an organ bath. We can apply drugs and examine their effects on the vessel function, and promising drugs may be progressed to experimentation in 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 number of animals needed for each experiment is calculated based on our previous experience and experiments, and will depend on the variable being examined. Using the typical amount of variation in our earlier experiments allows us to calculate the minimum numbers of animals used whilst ensuring that the results are statistically meaningful. For example, when looking at the differences in blood pressure between two types of mice, we have calculated that each group will need 24 mice.

Also, we have used our previous data from our annual returns of procedures to estimate the total numbers.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have used resources from the NC3Rs and Royal Veterinary College to help with our experimental design, and consulted with a statistician. To reduce numbers, it is important to reduce variability. To reduce variability, we will do the following:

- Where possible the same person will conduct the experiments, and information regarding which animals are in what treatment group will be hidden to avoid bias.
- A standard operating procedure will be followed for experiments and analysis
- We will use the same ages of mice

- Experiments will be conducted at the same time of day Animals will be randomly assigned to groups
- A randomised block design will be used

In addition, we will follow a sequential design that includes an interim review after we reach the half way point i.e. 12 animals per group. If at this time point the evidence is convincing, we can terminate the experiment and thus reduce the number of animals. This strategy has the potential to reduce the number of animals required by 15-25%.

There are important differences in male vs female biology that would significantly increase variability in our studies, therefore if we were to combine the use of male and female mice, this would greatly increase the number of mice needed. To avoid this, most of our studies will be performed in male animals. Experiments comparing the differences in female vs male biology will begin on a smaller scale and results will be used to direct future studies.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

At the end of the experiment, we will harvest as many tissues as possible. Tissues we don't use immediately will be frozen and stored for use later, or for sharing with other researchers.

When starting new studies where the effects of administering a substance are unknown, we will start by testing in a small pilot study group.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The majority of mice in this PPL will undergo minimally or non-invasive tests on their cardiovascular systems very similar to those done in humans, for example blood pressure, recording of heart electrical activity or blood tests, enabling us to precisely measure their cardiovascular health with little to no pain, suffering, distress or lasting harm. These may involved gently restraining the animals, and some mild discomfort following blood withdrawal. We will follow NC3Rs guidelines on blood collection techniques and volumes to ensure harm is minimised. For some experiments, like echocardiography (ultrasound scans of the heart), we will need to anaesthetise the animal to be able to take for these recordings.

This won't be for long and animals will recover quickly. Performing these tests will allow us to study the progression of disease, much like we would in a human.

To study how obesity causes diseases, mice may be fed a modified diet such as a high fat diet, but this will not cause any harm or distress. We will ensure that diets are palatable and all of their nutritional needs are met.

We will also investigate how exercise in obesity might treat diseases. For these experiments, some mice will be trained to exercise either on a treadmill or by swimming. To minimise stress and to help the animal acclimatise, the amount of exercise will be gradually increased. These approaches allow us to closely mimic human behaviour and the most popular exercises used by the general public, and they will not cause any lasting harm.

For some experiments, we will administer substances by injection or by oral gavage. This will allow us to investigate chemicals that may be important in the development or treatment of disease. Animals will experience mild, transient pain or discomfort, but no lasting harm. We will ensure we use the most appropriate and minimally invasive method, therefore causing the least amount of pain.

Some animals will be infected with a parasitic worm. However, the worms we use are not harmful, and do not normally result in any pain, suffering, distress or lasting harm.

In a small number of mice (~7%), a device that can record blood pressure and/or electrical activity in the heart will be surgically implanted. These animals will experience some discomfort for a few days after surgery and some mild to moderate pain which will be treated with pain killers, but they will make a full recovery. This is sometimes needed to allow us to take these measurements in live, unrestrained animals.

Why can’t you use animals that are less sentient?

Non-mammalian animals don't replicate the human cardiovascular and/or immune system to provide relevant results. As we study obesity, many of our mice will need to be fed a modified diet to induce obesity, and this will take time. Comparisons must always be made in the same age animals to reduce variation. Measures need to be taken throughout the animals life to track disease progression, therefore cannot be conducted under terminal anaesthesia.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Welfare of the animals will be monitored by trained technicians and researchers. A scoring system will be used to help identify any pain or suffering early and take appropriate measures such as administering pain killers. The scoring system will monitor changes such as weight, temperature, appearance, and heart rate. If these effects are seen and

either cannot be treated or do not resolve within a day, or if severe effects are seen, the animals will be humanely killed.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will make use of NC3Rs resources, PREPARE guidelines, LASA principles, and published guidelines such as the report by the Joint Working Group on Refinement on substance administration [1].

1. Morton DB, Jennings M, Buckwell A, et al. Refining procedures for the administration of substances. Laboratory Animals. 2001;35(1):1-41.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We have signed up for the NC3Rs newsletter, and will regularly check information on the website and attend relevant symposia and events where possible both externally and internally within our Institution.

85. The development of novel therapies (including cell and gene based approaches) for Parkinson's and Huntington's disease

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Stem cells/gene, Novel therapy, Immunogenicity (the ability of cells/tissues to provoke an immune response), Parkinson's disease, Huntington's disease

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?

There is currently no cure for Parkinson's disease (PD) and Huntington's disease (HD) or any way to stop it from getting worse. Our project is to show if new therapies, including gene and cell therapies, can improve symptoms and restore neural circuits in animal models of PD and HD.

Rejection is caused by the immune system identifying the transplant as foreign, triggering a response that will ultimately destroy the transplanted cells or gene. Our project is to understand how the human immune system respond to cell transplants and/or gene injections designed to treat aspects of PD and HD, then find ways to prevent them from being damaged by the human immune system.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Parkinson's Disease is the second most common neurodegenerative disorder affecting approximately 1% of those over 60 and is expected to become increasingly prevalent in an ageing society. Currently available therapies provide symptomatic relief for some of the movement problems, but to date, there are no disease modifying therapies for PD.

Huntington's disease is a rare genetic neurodegenerative disorder affecting approximately 2.71 people per 100,000 worldwide. It is a fatal condition that typically develops between the ages of 30 and 50 years old and progresses over a 20-year period. There is no cure for Parkinson's disease and Huntington's disease currently. It is important to find novel therapies for these patients that could better help treat their clinical features as well as slow down disease progression.

What outputs do you think you will see at the end of this project?

Publications and presentations. We anticipate publishing the results of these studies in peer- reviewed scientific journals. We also intend to present the findings of our work at local, national and international scientific meetings as well as to patient groups and at public meetings.

Novel data to support future funding applications.

We hope to use our data to design future clinical trials around our discoveries thus allowing for a translational aspect to the work. We are very motivated to develop clinically relevant potential therapeutic applications for patients with Parkinson's and Huntington's Disease.

The generation of new information around better understanding the neurobiological basis of Parkinson's and Huntington's Disease in vivo (studies done within living organisms).

The data produced by this project will potentially support patents application of these candidate therapies.

Who or what will benefit from these outputs, and how?

In the short-term these findings will benefit the ongoing research within the laboratory including developing new therapeutic agents and better understanding the immune response to gene therapies and transplants of stem cell derived products placed in the brain. In the medium-term this work will help to develop potential new therapies that could be used to treat Parkinson's and Huntington's disease as well as to better understand the need and type of immunosuppressive regimes required in such patients treated with gene and cell therapies.

In the long-term we hope that this work will lead to translational outputs in the form of clinical trials around new therapeutic agents.

How will you look to maximise the outputs of this work?

Collaborating- we will work closely with groups who have complementary knowledge using humanised mice (mice lacking their own immune system but given a human immune system) as a model to study immunogenicity as well as with groups working on novel therapies including cell and gene therapies and ways of delivering these to the Central nervous system (CNS).

Disseminating new knowledge - this will be done through presenting our work including negative results at local, national and international meetings and by publishing our work in peer reviewed journals as well as presenting findings to patient and public groups as appropriate.

Species and numbers of animals expected to be used

- Mice: 3350
- Rats: 500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use adult mice wild type littermates and sham transplants as appropriate for controls in our experiments. This will allow us to ascertain whether any effect seen during the experiments carried out is a true one and will thus avoid any false positives.

We will use adult immune-compromised mice that have been given a human immune system (humanised) to address the immune response of human cells within the mice in response to transplantation of stem cell derived products or gene injections. Humanisation is when human immune cells are introduced into the mice (by injection), they can survive and form a working immune system. This process is called engraftment. We have extensive experience of working with immune- compromised mice and know that they are the best type of mice to use for these sorts of experiments.

We will use adult transgenic mice as a model for studying Huntington's disease. We will use these mice to better understand the effect of novel therapies including cell and gene therapies in these diseased animal models.

We will use adult rats to model Parkinson's disease via a novel delivery system to transfer abnormal protein aggregates across the blood brain barrier and explore the possibility of validating existing or novel therapeutics. In short, preformed fibril (pff), a type of abnormal protein aggregate, will be injected intravenously then the pff will get into brain by penetrating the blood brain barrier and gradually cause some brain damages similar to Parkinson disease patient's. We will also use adult rats to model Parkinson's disease by injecting locally into the brain with neurotoxin such as 6-OHDA.

We will use adult rats to model Huntington's disease by injecting locally with a virally delivered mutant Huntingtin (mhtt) construct. By injecting mhtt expressing virus into the brain, we are hoping to make a Huntington's disease model.

Typically, what will be done to an animal used in your project?

Immunodeficient mice may undergo a combination of the following procedures depending on the experimental design.

We will use adult mice that lack an immune system of their own (such as NSG mice) to answer "To demonstrate the immunogenicity of transplanted cells/genes in humanised

animal models". Because these mice lack an immune system, they can be "humanised". That is - when human immune cells are transplanted into the mice (by injection), they can survive and form a working human immune system. This process is called engraftment. We have extensive experience of working with NSG mice and know that they are the best type of mice to use for these sorts of experiments.

To create the PD model, we inject a neurotoxin into the adult mouse/rat brain to mimic the damages in the patient's brain. This process is called lesioning. After lesioning, animals will undergo behavioural testing (such as walking on balance beam and grip strength) to find out if their behaviour is impaired, i.e. they have the symptoms of PD. If the disease modelling is successful, cell transplantation or gene injection (cell/gene therapy) will be carried out on those animals, aiming to reduce/eliminate the symptoms, which will be demonstrated by repeating the behavioural testing up to 6 times. Mice will receive one (for lesioning) and/or a second (for cell transplant) anaesthetics.

R6 mice are a transgenic line that carries part of a mutant form of the human Huntington gene and can be used to model HD. When R6 mice start to show symptoms of HD, they will undergo a battery of behavioural testing before and after the cell transplantation or gene injection to investigate any effects of these therapies. The behavioural testing will be repeated up to 6 times.

Rats may undergo a combination of the following procedures depending on the experimental design:

Rats will be injected intravenously (on a rare occasion up to three iv injections each under anaesthetic) with a type of abnormal protein aggregates (pff) or locally injected intracerebrally with neurotoxin or a virally delivered mutant Huntingtin (mhtt). Again, a battery of behavioural testing will be performed before and after injection to enable comparison of the effects of therapies. The behavioural assessments will be repeated maximum 6 times and the majority of rats will receive 2 anaesthetics (one for disease induction and one for injection of cells/genes).

What are the expected impacts and/or adverse effects for the animals during your project?

An estimated 20% of mice of strain NSG will show swelling around the hocks. Any animals experiencing swelling around their hocks may be given an altered enrichment, pain relief and/or anti- inflammatories in consultation with the NACWO and/or NVS.

No or minimal adverse effects are expected from transferring a human immune system (humanisation) of these animals other than the transient discomfort from the injection. Graft versus host disease (GvHD) is something that will occur over time and will be watched for as a side effect of the humanisation procedure. We have a great deal of experience of the time course of this happening.

Following stereotaxic injections (three-dimensional surgical technique that enables precise injection deep in the brain) of toxins, gene therapies or stem cells, our experience is that this induces no harmful lasting side effects, with animals returning to normal after recovery. The following adverse effects are expected post-operatively: animals may be subdued but show progressive improvement in response to stimuli. Over the period 24-96h, animals may show reduced appetite for food and water, which is expected to result in minor weight loss (less than 5-10% of their pre-operative weight) within this 4-day period. Minor dehydration may therefore also occur and will be alleviated by addition of mash or

gel to the cages.

For the rats used in the procedure involving intravenous administration of abnormal protein aggregates, this will be conducted under general anaesthesia, which normally last between 5-10 minutes. Due to the short time that these animals will be under anaesthesia, we expect them to recover quickly with no more than transient discomfort and without lasting harm from the anaesthetic procedure itself. From 6 months after the initial intravenous administration of abnormal protein aggregates, it is possible that some animals may start to develop Parkinson's disease-related phenotypes, such as deficits in the olfactory (the sense of smell), motor, and cognitive functions. As a result, animals can show signs of reduced activity, abnormal gait (manner of walking), as well as changes in body weight. While we hope such deficits to slowly progress over the next few months (up to 15 months of age), they will not cause any pain to the animals, and will not interfere with their ability to move, drink or eat freely.

R6 mouse strains are expected to progressively show adverse effects associated with a mild to moderate severity category. The majority (~80%) of animals bred and maintained under this protocol are expected to remain within or below the mild severity criteria. Approximately 20% of all animals are expected to develop a neurodegenerative phenotype reaching 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)?

Mice Mild-10%
Moderate-90% Rats
Mild-10% Moderate-90%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Neurodegenerative diseases such as PD and HD are associated with widespread pathology and many neurological and cognitive deficits, necessitating whole system animal studies, although these can be supplemented by techniques such as in vitro (performed in a test tube, culture dish or elsewhere outside of a living organism) slice cultures. Some information about the networks that underlie specific clinical aspects of the disease can be gathered from the patients, using techniques such as functional imaging as well as post mortem studies but trying to treat them necessitates studies in animal models.

Animal models are needed to study causality and disease progression in ways unavailable with patients. For example, we cannot interfere with the drug regimens used to treat

patients clinically nor can we undertake precise mechanistic and therapeutic studies with novel agents without recourse to animal models to show survival, safety and functional efficacy.

Thus the experiments undertaken in the licence can really only be done in animal models of disease given that we plan to: (a) mimic the clinical scenario as closely as possible in the complex mammalian brain and how it responds to agents that are designed to work by either rescuing or replacing networks of degenerating neurons; (b) study the efficacy of therapies either across the pathology of the whole brain or in selective populations and networks of connected cells; (c) study the effects of therapies on a range of animal behaviours over time including motor and cognitive deficits; (d) document the possible side-effects of any such therapies including weight changes, etc and e) show that the cell and gene therapy survives and innervates or distributes over the volume of brain needed for patients.

Which non-animal alternatives did you consider for use in this project?

Growing stem cells derived from patient's skin biopsies will allow in vitro studies to replace some that involve animal tissue, and developments in biochemical analysis of patient's samples such as blood and cerebrospinal fluid are yielding more information, supplementing that from animal studies. Some in vitro modelling using embryonic stem cells and relevant derivatives have been used to study human immune responses along with in vitro human foetal tissue studies for testing new potential therapies.

Why were they not suitable?

This in vitro work is a useful starting point but ultimately cannot be used to study how the therapies work within the diseased brain and affect the systemic human immune system. This can only be done using animal models of disease and humanised 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?

This number has been estimated from our extensive experience of doing this kind of work. We have estimated that 350 mice will be required for "rederiving" (i.e. establishing a new colony of mice from frozen eggs and/or sperm) any further strains of mice we need for this project. Up to 3000 mice will then be used to answer the experimental questions.

We will use up to about 500 wildtype rats for experiments on Parkinson's and Huntington's disease model.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

During the experimental design phase, we (i) sought guidance from the NC3R's

experimental design assistant on-line tool and (ii) reached out to collaborators who have extensive experience of the specific animal models we are using. In addition, we have in house experience of neurotoxic lesioning and cell transplantation, and our number estimates are based, in part, on these data. Our experimental design has also been informed by effect sizes seen in vitro and similar work done in the past or published.

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 animal usage, we will:
Breed animals as efficiently as possible - attempting throughout to match breeding with experimental needs as closely as possible. This will require careful experimental planning.

Throughout the project we will perform our experiments on small groups of animals (3-5 animals at a time), in pilot studies, and will review the results prior to extending our work to larger numbers and thus we will adapt our work accordingly.

Share animals with local collaborators - this is particularly relevant for NSGs where there are a number of groups locally using this animal model. This is something we routinely do on our current licence.

We will consider placing mice into experimental groups randomly and that treatments are given 'blind'. In that way, the experimental results are analysed blindly as well to avoid any bias when drawing conclusion.

In certain experiments we could do bilateral stereotaxic surgery placing the experimental stem cells in one side of the brain and a sham injection on the other side such that a mouse could act as it's own control. This will reduce the numbers of mice being needed for certain 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.

Transgenic mice will be employed in some studies as they contain the genes responsible for unique human disorders, such as HD. This enables studies which more completely recapitulate the pathology and behavioural deficits of the human disorder.

PD or HD rat/mice models can be also achieved by acute surgery. Acute surgical lesioning may involve more than one lesion (e.g. bilateral injections), but this is limited to a single surgery session of lesioning. Acute lesions may actually have either an acute effect (whereby lesions are created rapidly over a short period of time) or a progressive effect (whereby lesions develop slowly over time in response to an initial insult) mimicking the progressive nature of PD and HD neurodegeneration. An example of an acute lesion is

one created through the administration of neurotoxin which takes almost immediate effect and for instance, aims to irreversibly remove the dopamine input to a certain part of the brain akin to that seen in advanced PD. An example of a progressive lesion is through the administration of viral vectors by a single injection to the brain, which leads chronically over time to the progressive accumulation of pathological proteins in cells and so mimics the progressive cell loss.

Other experimental work can be best done in rats (particularly cellular therapy strategies) given the long history of their use in our studies and the better described behavioural repertoire. Typically, the lesions are either acute or chronic and the animals display specific behavioural deficits that will be ameliorated if the novel therapeutic including dopamine cell therapies work to restore or reverse the pathology seen in these models. For example, from 6 months after the initial intravenous administration of abnormal protein aggregates, it is possible that some animals will start to develop Parkinson's disease-related phenotypes, such as deficits in the olfactory, motor, and cognitive functions. As a result, animals can show signs of reduced activity, abnormal gait, as well as changes in body weight. While we hope such deficits to slowly progress over the next few months (up to 15 months of age), they shall not cause any pain to the animals, and shall not interfere with their ability to move, drink, or eat freely.

We will use adult mice that lack an immune system of their own to study the immunogenicity of novel therapies by humanising them. That is - when human immune cells are transplanted into the mice (by injection), they can survive and form a working human immune system. This process is called engraftment.

Why can’t you use animals that are less sentient?

Rodent models are the least sentient organism still able to reasonably mimic the human neurodegenerative diseases we are studying, with a brain which is anatomically similar to the human brain. Mice and rats differ both in their behavioural capacities and in the experimental practicalities as a consequence of their differing brain sizes; although the basic approach remains similar for both species. Which species is used in each experiment will be determined to ensure that we maximise the probability of obtaining clear, valid and reproducible results whilst causing minimum distress to the animal.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All animals used on this project will be monitored daily for any signs of ill health or distress. Particular care will be taken after surgery, with increased frequency of observation over the first few hours post- operatively, and through the use of pain medication which could be provided in the way of flavoured jelly (or such-like) that can be self-administered by the mouse. Immediately post-operatively mice will be kept warm and then will be housed in cages with additional soft bedding.

Throughout our project, we will aim to improve the quality of life for our animals – for example by providing enriched environment, acclimatisation and housing mice in social groups whenever possible and by avoiding single housing (apart from in exceptional circumstances, which will be time limited).

Any animals experiencing swelling around their hocks may be given an altered enrichment, pain relief and/or anti-inflammatories in consultation with the NACWO and/or NVS.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We are aware of, and will always aim to adhere to, good practice guidelines (as given by the Laboratory Animal Science Association; LASA). We will ensure that we follow the LASA guidelines for performing aseptic surgery (as given in the updated 2017 document) – such as writing a pre-operative plan, administering pain medication and fluids as required and most importantly ensuring that the procedure is performed by a researcher who is fully trained.

We are also aware of the PREPARE (Planning research and experimental procedures on Animals: recommendations for excellence) and ARRIVE Guidelines (Animal research: Reporting of in vivo Experiments, version 2.0). PREPARE guidelines are for planning animal experiments as improved reporting, although important itself, can't improve the quality, reproducibility and translatability of animal studies. These guidelines are designed to complement ARRIVE guidelines that are a checklist of information to include in publications describing animal research. It includes their reporting guidelines around the importance of information on experimental design and statistical analyses and strategies to minimise bias and how to report in publications.

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 by speaking to colleagues locally (including veterinary surgeons, senior animal house technicians etc) and by reading information sent to us by the local licencing department. We will also ensure that we stay up to date by visiting relevant websites (including that of the NC3Rs and Norecopa) and by attending relevant conferences and training events.

As a project team we will meet regularly to ensure that we are adhering to the 3R guidelines and implementing any changes effectively.

86. Immunity against emerging pathogens and 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

Vaccines, Infectious diseases, Immunology, Paediatric diseases

Animal types	Life stages
Mice	adult, juvenile, aged, embryo, neonate, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to study how the immune system responds to infectious disease and subsequent vaccination, which is important to the development of new vaccines and their efficacy.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 twenty-first century, newly emerging infectious diseases have continuously threatened public health and caused outbreaks of global concern. This has been highlighted by the recent COVID-19 pandemic which caused a catastrophic effect on the world’s healthcare system and the global economy. There are a number of new and dangerous pathogens with recognised pandemic potential but there are no specific treatments or vaccines. Our aim is to develop and test the novel vaccines against these infectious diseases with the primary goal of enhancing human health.

We are also interested in understanding immune responses involved in infectious diseases.

What outputs do you think you will see at the end of this project?

The outputs that we will see at the end of this project are:
Development of new vaccines or their components against several infectious diseases

New information in terms of how these vaccines will provide protection.

New information in terms of how these vaccines will be impacted by other factors such as genes and the immune system.

Publications in scientific journals.

Who or what will benefit from these outputs, and how?

In the short to medium term, we expect the scientific community who are interested in infectious diseases to benefit from our findings.

In the medium to long term, we expect pharmaceutical companies and ultimately clinicians and those patients, which are mainly infant, young children and elderly, affected by infectious diseases to benefit from new vaccines developed as a result of our findings.

How will you look to maximise the outputs of this work?

We will maximise the outputs of this work by publication in open-access journals, presentation at scientific meetings, making resources available to other researchers (e.g data, animals and tissues).

Species and numbers of animals expected to be used

- Mice: 13000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are good candidates for immunological studies and therefore for assessing vaccines as mouse is the least sentient animal that is genetically and physiologically similar to the human. In this project, we will use mice from different genetic backgrounds. For studies aiming at assessing how the immune system responds to a specific vaccine, non-genetically altered (wild-type) mice will be used. For certain specific studies aiming at better understanding the role played by a specific cell population in the immune response to a vaccine, genetically altered strains will be required.

For vaccination studies, we will use mice at ages that are clinically relevant in vaccinology. Juvenile and adult mice will be used to model vaccination in infants and adults respectively. Aged mice will be used to study long-term maintenance of the immune

response and mimic vaccination in elderly populations.

Typically, what will be done to an animal used in your project?

Genetically altered mice will be bred using conventional methods of mating. The genetic mutations the mice carry are not expected to cause any harms and the mice will behave and function normally. The majority of the mice will be purchased from commercial sources.

Some mice will receive vaccines that are expected to be effective against a viral or bacterial infectious disease. The vaccines might be given in conjunction with an adjuvant that is not expected to cause any irritation or lesions to the skin where the injection is given. Typically, we use the intramuscular route into the hind limb of the animal as this is the preferred route in the human. However, we may use alternative routes of administration when trying to target the immune response to specific sites. This will be intranasal/ocular/oral for respiratory diseases, rectal/vaginal for diseases of the genital tract, topical application for skin diseases such as those transmitted by mosquito bites and orally for gastrointestinal diseases.

To check that a vaccine triggers an immune response in the body, which would suggest it is effective, and in order to track this response, animals will undergo blood sampling at regular intervals during the experiment.

Some mice will be exposed to a low dose of irradiation. This will delete the bone marrow and then they will be given bone marrow from another strain of mouse to replace the depleted bone marrow. This process is like bone marrow transfusion in humans which is used to treat blood and immune systems diseases which affect the bone marrow and track the specific parts of the immune system. Antibiotics may be given to prevent opportunistic infection, typically for up to 28 days around the time of irradiation.

Some mice will also receive substances or cells that will help us deplete or enrich certain types of cells. Other substances such as cell markers, monoclonal antibodies or tracers may also be given as will antibiotics as a therapy.

As the immune system and therefore the efficacy of vaccines may be different the older person, we will use older mice in our experiments. Mice may be aged until they are 24 months of age.

At the end of the experiment all animals will be humanely killed, with tissues and blood collected post mortem for analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

Vaccination

Vaccines are not expected to have any adverse effects that are more than transient in nature. The vaccines or the control substances are typically given by the intramuscular route into the hind limb, like humans receiving injections into the upper arm. However, unlike the human mice will be under general anaesthesia to reduce the pain involved. Mice may experience a short period, typically no more than 24 hours, where they show signs of general malaise such as hunched posture, piloerection, and lethargy due to over stimulation of the immune system. This is like the human response to the annual flu

vaccine.

Sublethal whole body irradiation

Mice will undergo sublethal irradiation, meaning that they will be irradiated with a non-lethal dose, usually slightly lower than a lethal dose, to create bone marrow damage. During this process, their immune system will be depleted for a short period of time, until they are transplanted a new bone marrow that will replenish the immune system. In the time frame between bone marrow damage and transplant, these mice will be susceptible to infection. Adverse effects will include transient weight loss of up to 15% between days 7 and 10 post irradiation, but which is self-resolving by day 14 post irradiation.

Expected severity categories and the 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 would expect the animals to experience the following severities and proportion: 22% Subthreshold, 70% mild and 8% 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?

Vaccination against a specific infection triggers the immune system such that the body is further protected against this disease. Immune response to vaccination involves a network of cells, organs, and tissues in the whole body, and therefore cannot mimic the full immune responses in vitro. Mice are good candidates for this purpose, as they are the least sentient animal that is genetically and physiologically similar to the human and are the most characterised species for detailed immunological analysis. There is also need to assess the safety of new vaccines in mice before these are administered into human.

Which non-animal alternatives did you consider for use in this project?

We use cell cultures (in vitro assays) to test the expression of our vaccines in mammalian cells before we move to in vivo testing.

Why were they not suitable?

Whilst in vitro assays or cell studies can be used to assess vaccine efficacy, they are not a true representative of the complex interactions that occur in the whole organisms. Therefore, they are a vital tool in our studies prior to moving into the mammal, but not a suitable replacement.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 several programmes to develop new vaccines against emerging pathogens and infectious diseases funded by UK government and charity organisations. We have estimated the number of animals based on the number of ongoing programmes and other potential programmes where we will be developing new vaccines during this project. Sample sizes for our experiments are estimated from our past experiments which showed that we need group size of 5-6 to achieve the quality of results we need. We have also used our annual return of procedures data to estimate the number of animals that we have used from the group each year. We will apply the principles of experimental design at each stage of work to achieve reliable results and avoid unnecessarily repeating experiments. We will (1) appropriate selection of experimental groups (controls and dose levels), (2) maximising the data output from the animals by using our established immunoassay protocols, (3).

Minimise the variability by harmonising experimental conditions and techniques within the group. (4) determination of group sizes based on the data that exist from previous work, (5) use of small groups for pilot studies when testing new vaccine platform or adjuvant.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have taken number of steps during the experimental design phase to reduce the number of animals being used in this project such as:
Ensuring that each experiment will only have to be performed once where possible.

This is achieved by a refined study design with a high translatability and an emphasis on reproducibility.

Refinement of experimental designs using randomisation, blinding, and implementation of measures to reduce bias as relevant study design and statistical analysis are key to ensure reduction.

Use of the experimental design assistant (a tool from NC3Rs) to ensure the best design for our studies.

NC3R’s EDA was used to get support for randomisation, blinding, and sample size calculations as well as to provide practical information to improve knowledge of experimental design.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

In order to optimise our experimental design, we are planning to use pilot studies.

Pilot studies involve a low number of animals per group (for instance n=3) and are typically useful to narrow down the number of conditions that will be tested later on in an experimental setting on larger cohorts. For example, in a study where we plan to test 3 doses and 2 dose regimens (prime only and prime + boost), we will consider using a pilot study to compare the different doses and dose regimens and find the best conditions for the follow-up study that will involve a larger number of animals. In a scenario where certain assays/methods have not been established or optimized yet, pilot studies constitute an ideal opportunity to provide a few samples that can then be used for this purpose. We will also consider using pilot studies to establish the variability of the methods that are planned to be used in the study.

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.

Cryopreservation of colonies not required in the short term will be considered.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will try to minimise animal stress and suffering to the greatest extent possible.

We are acutely aware of the effect stress may have on our results and strive to keep each animal in an optimal state. Therefore, we will ensure that handling methods will follow best practice. Typically, we will avoid picking up mice by the tail, which has been shown to induce aversion and high anxiety. Instead, where possible, mice will be picked up using a non-aversive method that promotes a positive response to human contact, such as tunnel handling and cupping method. Where possible we will also train the animals to cooperate with procedures to minimise any distress as evidence suggests that pain and suffering can alter an animal’s behaviour, physiology and immunology, which can lead to variation in experimental results, impairing both reliability and repeatability. Animals will be held in bio-secure caging to reduce the risk of opportunistic pathogens. We will ensure that appropriate anaesthesia is used to minimise pain and follow guidance on single use needles at all times.

Why can’t you use animals that are less sentient?

Less sentient animals such as fish and worms do not have a complete immune system that is like that of the human, which is vital for our work, therefore we use the species that is the least sentient model.

We cannot use animals under terminal anaesthesia as the immunogenicity of our vaccines need time to develop.

How will you refine the procedures you're using to 738inimize the welfare costs (harms) for the animals?

Vaccination in adult animals

Our vaccination platforms have been extensively tested in both mice and humans and are not expected to cause any adverse effects other than occasionally a mild transient general malaise for up to 24 hours. All animals will be monitored for at least 24 hours post vaccination and additional monitoring points will be added to animals showing clinical signs.

Sublethal whole body irradiation

Mice undergoing sublethal whole body irradiation prior to bone marrow transplant might present with common signs of systemic reaction and/or infection such as piloerection, lack of grooming, altered behaviour, reduced movement or lethargy, laboured breathing, and/or weight loss. Actions will be taken to reduce the burden on the animals: split dose irradiation, supplemental nutrition with milk powder before and after irradiation, administration of prophylactic antibiotics under advice from a Named Veterinarian Surgeon (NVS). Mice will be carefully monitored daily for 14 days following irradiation and weekly until they return to a normal state. Body weight will also be monitored and mice showing a body weight loss greater than 15% will be culled immediately using a schedule 1 method. A similar approach will be used to alleviate the burden on mice undergoing administration of bioproducts (cells, serum, antibodies) or diverse substances (for instance DNA markers or signalling modifiers).

Ageing animals

Ageing animals will be carefully monitored by staff trained to work with ageing animals.

Group sizes in ageing experiments will be increased to accommodate for loss of animals and to avoid single housing due to animal losses due to old age.

Longer drinking spouts will be used, and animals will be monitored for adverse effects such as changes in weight, dermatitis, piloerection, paleness, changes in mobility, lumps, eye defects, abnormal respiration, or stools. If these are observed animals will be treated accordingly and closely monitored. Animals will be humanely killed before displaying severe symptoms.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use the following published best practice guidance to ensure experiments are conducted in the most refined way.

LASA guidelines for best practice on dosing volumes and regimens (https://www.lasa.co.uk/current_publications/)
www.nc3rs.org.uk for guidelines on the 3R's, the experimental design assistant and for welfare considerations during experiments.
https://arriveguidelines.org/ provide very detailed guidelines on study design and conduct

to ensure reproducibility of the data compliance to the 3Rs.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We attend regular 3R events at the establishment including regular welfare meetings where new initiatives are discussed. Our establishment has a dedicated 3R's committee from which our Named Information Officer disseminates information.

We also subscribe to both the NC3R's and RSPCA animals in science newsletters. We will regularly perform internet searches for information on any new alternatives to animals for our research and for any new refinements that we can implement in our projects.

87. Cell behaviours in morphogenesis of anatomical structures during 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

Development, Embryo, Morphogenesis, Cell biology, Regeneration

Animal types	Life stages
Mice	adult, pregnant, embryo, 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 use mice to understand how cell behaviours, especially cell proliferation, cell movement, and cell shape change, produce the essential anatomical structures in our bodies and thereby provide understanding that will be foundational to diagnosing, repairing or regenerating such structures in normal development, or following injury or 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?

Biological science has discovered a great deal about genes and how they regulate one another and proteins, but the link between genes and physical outcomes at the level of tissues is what cells do as a result of the genes that they and their neighbours have activated. Our knowledge of cellular activities is still very limited and so work under this

project is important to build it, establishing cause and effect between genes and their phenotypic (i.e., physically observable) outcomes.

What outputs do you think you will see at the end of this project?

This project is designed to generate fundamental insights into the basic biology of embryonic development and later wound repair and regeneration in animals related to humans. Its outputs will therefore include new knowledge about how particular structures form. These range from tiny tubes in the inner ear that enable us to hear sounds to the walls that separate the chambers of our heart, to the cartilage that shapes our jaws. Some processes will be shared between these structures while others may be unique to each one, but the principles behind their formation and the defined and limited repertoire of cell behaviours involved in tissue growth will form a unified project and body of knowledge. Outputs will include peer-reviewed publications, scientific datasets in public repositories, international collaboration and exchange, educational activities for students in higher education and outreach to schools and the general public.

Who or what will benefit from these outputs, and how?

Short term impacts will be primarily academic since this is very basic science (i.e. aimed at fundamental understanding for application in the long-term). However, they will also include public engagement and educational activities that will enrich people's appreciation of the wonders of developmental biology. Longer term impacts will be the precise tailoring of organoids - model tissues used for investigating many aspect of biology and pharmacology - and other tissue engineering applications where understanding of biological morphogenesis will complement the use of artificial "scaffolds" for repair/regeneration. Since organoids provide excellent models for disease mechanisms and drug action and are therefore already in use by pharmaceutical and biotechnology industries, this project will also provide translational opportunities through there improvement and refinement.

How will you look to maximise the outputs of this work?

Outputs from this work will include publication of primary and review articles in the open-access peer- reviewed literature, aiming for high impact journals with large readership and media coverage.

Knowledge will be disseminated through presentations at academic conferences and public engagement, both in the UK and internationally, and through hosting visiting students and researchers to share expertise. Presentation will include guidance on more and less successful/fruitful methods and technologies, protocols and instruments.

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.

Investigating the formation of tissue structures in embryonic development absolutely requires that real embryos are investigated. Mice are excellent models for this type of investigation since most features of their anatomy are highly similar to those of humans. Not only do their small size, rapid development and rapid breeding provide experimental convenience, their well-understood genetics and the availability of many variant, mutant and engineered lines (e.g. mouse models of Down syndrome that have the same craniofacial and heart defects as Down syndrome babies) constitute an unparalleled set of tools for observation and perturbation needed for the experiments in this project. Embryos will be investigated in mid-gestation, roughly day 10 to day 16 of a 19- to 21-day pregnancy, which is when the detailed anatomy and organs take shape.

Typically, what will be done to an animal used in your project?

Most procedures in this project will involve breeding lines of genetically engineered or otherwise mutant mice. This will be because the mice express proteins that enable certain cells to be labelled so they can be tracked or more easily observed under a microscope than unlabelled cells (so-called reporter lines), or because the mice display a predictably dysmorphology (malformation) that models a human inherited birth defect. Sometimes the mice will be injected with a compound that activates a genetic labelling process, or directly labels cells undergoing a process of interest (e.g. synthesising DNA before dividing). Such compounds are used routinely by many researchers and cause no or minimal harm, so only disturbance to the animals is the injection procedure itself.

What are the expected impacts and/or adverse effects for the animals during your project?

Mouse reporter lines are physically normal despite expressing fluorescent proteins unconditionally or following chemical activation, so usually experience no harm (with a formal classification as "sub- threshold"). No impacts or adverse effects are expected for this type of animal.

Dysmorphic mice are usually kept as fertile and viable "heterozygotes" so that one of the two versions of the relevant gene masks the effects of the other dysmorphology-causing version. Usually these mice are completely normal or have minor abnormalities that have no detectable effects on the animal's welfare. Sometimes the masking is imperfect so that the heterozygote has mild abnormalities (e.g. a slight tremor that does not affect normal behaviour, feeding or breeding). Thus, there may be some mild impact on the animals carrying heterozygous mutations.

Occasionally, the crossing of two lines to test combinations of mutations might lead to more severe abnormalities, and as long as these do not affect viability, it may be necessary for these to be kept for analysis, although in general, the project will be directed at detecting these at embryonic or perinatal stages, at which point they will be humanely killed so that there is no harm to the animals at later stages.

Occasionally adverse effects such as weight loss have been experienced during and after dosing with tamoxifen, one of the reagents to be used to induce conditional mutation. From past experience, it is not expected that the application of induction agents in food and water will have any adverse effects. However, if the taste is aversive, then the mice may lose weight due to reduced food intake, or lose condition due to dehydration. There is also a very small risk of damage to the oesophagus during oral gavage (<1%). Tamoxifen can cause some adverse effects in juvenile male mice, namely reduction intestes size. Mice do not seem to be in any pain or discomfort. In addition, tamoxifen may cause some partial

herniation in the scrotal/inguinal region in a small number of animals, this is due to weakening of the abdominal muscles. This appears to be non-painful and causes no distress.

Tamoxifen injection into pregnant females can cause issues with littering down, with the risk of retained embryos.

Expected severity categories and the 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: 5% moderate, 10% mild, 85% sub-threshold. Note that most experiments under this project will be conducted on isolated tissues from Schedule 1- killed embryos.

We anticipate that the establishment of new genetically modified lines will lead to some unanticipated phenotypes, which will need to be carefully monitored. For these lines animals are culled prior to overt signs of distress.

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 biology of real tissue and organ formation and repair absolutely requires that real tissue is studied. Animals are therefore the only meaningful and ethical way to study these processes under physiological conditions.

Which non-animal alternatives did you consider for use in this project?

No non-animal alternatives exist for this project, even in principle. The lab has and will continue to use organoids to complement the studies in this project, but these definitively do not match the physiological processes that provide precision development of real tissues and organs. Many of the experiments that will be done associated with animals used for this project will not, formally speaking, be animal procedures because they will be conducted using tissue or organ explants following humane killing of normal wild type animals or embryos. For such experiments, however, there is no alternative source of material.

Why were they not suitable?

Organoids are impressive at one level but at the same time are still poor simulacra of real development, particularly when it comes to their architecture, which is the subject of this

project.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimate of number of animals to be used is based on prior experience with these protocols, both in my own group and that of the several others in my department doing similar work for over the past 15 years.

The majority of the mice will be used for breeding. Although we will not perform quantitative tests on our live mice, we will on the tissue collected from the animals that we are using under this licence.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

For defined experiments within this project, we will use the NC3Rs Experimental Design Assistant, which we have benefitted from in previous projects (including a project funded by NC3Rs itself) to design our experiments and help do power calculations. We will also ensure that following pilot experiments, we make sure to perform well-powered studies that do not become under-powered through typical attrition experienced in real-life experimental projects. This involves using a safety margin of higher numbers than the Power calculation minimum suggests.
For the majority of our experiments, we will use littermate (sibling) controls to minimise variability between animals. We will use outbred strains, such as CD1s, that have larger litters to facilitate this and to minimise the number of animals needed. Samples sizes for our experiments will be estimated from past experiments, and using power calculations, with minimal numbers of animals used whilst ensuring that results are statistically meaningful and reproducible.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Where possible we will use outbred strains that have large litters, thus reducing the number of animals needed to achieve sufficient observations on the relevant micro-stages and genotypes. (This will not be at the expense of using sufficient litters for good biological independence of observations, for which our standard is to use at least three different litters and n of at least 5.)

The other main measure taken that reduces the number of animals is that when morphogenesis is investigated in particular tissues, other tissues are made available to PIs in the Centre and, complementarily, we are able to use material surplus to colleagues' needs from their projects if appropriate for this project. Since the Centre focusses on development and tissue engineering and is highly collaborative, many of the genetically-modified mice we use are common for studies on other organs.

Additionally, to reduce the number of animals used, wherever possible multiple tissues and organs are studied from the same animal by different members of the group.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice are the main animal model used since they are the mammalian species most appropriate for studies based on genetic analysis. Their small size (making housing numbers feasible), rapid breeding (enabling new strains and crosses to be made in a reasonable time), deep knowledge-base for development and the extensive international resources of genetic-modifications together make them the only species for the proposed research. The common other developmental animal models used such as avians or fish do not have many of the mammalian-specific organs or organ anatomy, such as molar and incisor teeth, tongue, salivary glands, four-chambered heart, cochlea, middle ear, skeletal elements, etc.

Suffering is minimised by placing the animals in as stress free as possible environment – standard cages with plenty of spaces to hide, no overcrowding, and placing with suitable companions. Most of our procedures are sub-threshold or mild, which produce no or minimal transient discomfort to the animal. Most of the remainder of our procedures are mild (e.g. tamoxifen injection) with a small proportion being moderate (e.g. breeding of a strain with a chronic mild head tremor that has no other effect on normal health or behaviours) and all stress and pain is appropriately monitored and minimized in degree and duration throughout.

Why can’t you use animals that are less sentient?

Since most of the procedures involve breeding or and development during gestation, adult fertile animals must be used, which means that life stages that are less mature and therefore less sentient than what we already propose cannot be used.

Less sentient species would be frogs or fish, but these have organ morphogenesis that is less relevant to mammalian anatomy than do mice (for example, two-chambered rather than four-chambered hearts, primitive tooth structure, non-hollow neurulation, etc.). For mutant lines that model human dyspmorphology syndromes, these other species are often less faithful as models or simply do not exist.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animal lines with inborn risk of birth defects will be kept as normal “heterozygotes” (i.e. with the relevant gene mutation effects masked by the presence of a normal copy of the same gene) so that as a procedure it is sub-threshold. Embryos will be collected mostly at

pre-mid-gestation and therefore pre-sentient stages. Particular lines known to exhibit phenotypes as heterozygotes (e.g. the CelsrCrsh/+ mouse) will be monitored at increased frequency for abnormal behaviour or failure to thrive (including poor feeding or breeding) especially when crossed with other lines or into different backgrounds.When administering substances, as needed for some experiments, minimal doses will be used to avoid adverse side-effects and animals will be monitored for adverse effects.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Close attention will be paid to the literature around the particular genetically altered and similar lines to identify any potential welfare issues. Similarly, general good practice will be followed by attending to advice from our animal services department, the Named Vet and the applicant's departmental mouse User Group, which has regular meetings where such things are discussed. All new members of the lab will be partnered with more experienced peers to make sure that best practices are followed, first ensuring proper training but thereafter to ensure proper implementation of learnings until genuine proficiency in them is achieved. We will follow published guidelines such as the PREPARE guidelines and consult current published references including Animals in Science Regulation Unit (ASRU) Guidance and the Code of Practice for the Housing and Care of Animals Bred, Supplied or Used for ScientificPurposes Guidance on Breeding Protocols for Genetically Modified Mice.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our animal services department regularly distributes information around animal welfare including seminars and classes around current and new practices for 3Rs. Additionally, we regularly monitor projects funded by the National Centre for the 3 Rs and read its regularly issued newsletter. Finally, the mouse User Group in my department is an excellent peer group for exchange of information in this area. Good new techniques are always advantageous for reliable experimental results and so will be adopted whenever and as soon as is possible consistent with the conduct of the project as a whole.

88. Cellular mechanisms of circadian timekeeping

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Circadian rhythms, Biological clock, Sleep/wake cycle

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?

There is a biological clock, or circadian rhythm, within every cell of the human body that controls major aspects of our physiology, such as when we sleep and how we breakdown food. We know that this body clock is important for health, since when it goes wrong or gets out of synch with the day/night cycle (as in jet lag or shift work), people are more likely to suffer from conditions such as diabetes, cardiovascular disease and various forms of cancer. Therefore, we want to understand the cogs and gears that allow this daily biological clock mechanism to function within each of our cells. To do this we also must understand how it communicates with neighbouring cells, and how our physiology is tuned with the external cycle of day and night.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Healthy aging is linked with robust daily rhythms in our physiology and behaviours, such as the sleep/wake cycle, that are driven by internal circadian (about daily) clocks. Disruption of our natural circadian rhythms, as occurs during shiftwork and increases with age, is linked with a wide range of diseases such as neurodegeneration, diabetes, various

cancers and cardiovascular disease. To understand why, and help prevent disease, it is imperative that we understand what makes our internal circadian clocks tick and learn how they interact with many different aspects of our biology.

Because circadian timing and 'clock genes' are very similar between mice and humans, the experimental use of mice allows us to glean insights and test hypotheses that would not be possible if we relied solely on cultured cells and would be impractical or unethical to perform in humans.

What outputs do you think you will see at the end of this project?

Elucidating the fundamental processes that underpin the cellular circadian clockwork will help us to understand the complex interaction between lifestyle, our genes, aging and human health.

Furthermore, we hope our research will reveal potential targets for therapeutic intervention and management of sleep and other clock-related disorders, such as jet-leg and shift-work. Finally, we expect this work to extend our understanding of the mechanisms of brain disorders that have been linked to clock disruption, such as Alzheimer’s disease. In turn, this may lead to novel ways of preventing, slowing, or reversing these disorders.

Who or what will benefit from these outputs, and how?

Our research will principally benefit four groups:
Short term:
Research performed by other biomedical scientists and clinicians will benefit from understanding the new mechanisms that our research reveals, for example, by allowing experimental designs and interpretations to be refined in order to account for circadian regulation. An added benefit is that, by performing experiments at the biological time-of-day when the greatest effect is expected, future experimental animal numbers can be reduced as fewer experimental animals will be needed to detect a significant difference in outcome.

Through our proactive public engagement, the general public will benefit from understanding the importance of circadian rhythms to human health based on understanding gleaned from our experiments in mice. This will allow individuals to make informed lifestyle adjustments in order to facilitate healthy aging.

Medium-to-long term:
Shift workers comprise 15% of the UK work force. This group will benefit from our work through the application of interventional strategies designed to ameliorate the adverse effects of shift work on short- and long-term health, that will first be tested in mice and then humans via our clinical collaborations.

The efficacy and side effects of many drugs and surgeries varies with time-of-day, but understanding of circadian regulation is not sufficiently advanced that this can be predicted in advance. Our work aims to fill this knowledge gap so that, in future, treatment of diseases and acute injuries will be tailored to work with each patient's circadian rhythms, not against them.

How will you look to maximise the outputs of this work?

Research findings (positive and negative) will be made available to other scientists through

publication on pre-print servers, in open-access journals and by participation at scientific conferences and meetings.

Public engagement will occur through press releases, media interviews and science exhibitions. Clinical audiences will be engaged through targeted presentations at relevant meetings and collaborations.

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.

To be able to better prevent and treat human disease, we need to understand the fundamental mechanisms of the daily biological clock in every human cell, and how it functions in our bodies. The vast majority of our research occurs in cultured cells in vitro without requiring animals. Ultimately though, living animals and their tissues must be used to test the relevance of our research, as the health-relevant, biological end-points we seek to comprehend (circadian rhythms in human physiology and behaviour, sleep and wakefulness) are properties of the intact brain and body and so only occur in living organisms. The adult mouse is the simplest model organism that recapitulates all the essential elements of human circadian physiology, including the natural changes that occur as mammals age.

Typically, what will be done to an animal used in your project?

The typical experience for a mouse on our experimental protocols is to be individually housed in cages within environmentally controlled cabinets over several weeks, where food and water bottles are replaced weekly, with visual inspections occurring several times/week and remote inspection of locomotor activity occurring at least daily. This allows any potential welfare issues to be identified as they arise.

Each cage is situated so that mice can smell and hear their neighbours, and includes a running wheel and/or other environmental enrichment. This allows each mouse to express its natural daily rhythm of rest/activity, fast/feeding, sleep/wake under non-stressful conditions. Particular efforts are taken to maintain animal health and welfare during these experiments because physiological stress can disrupt endogenous circadian rhythms, and thereby interfere with our experimental objectives.

In a typical experiment, during week 1, animals would normally be maintained under daily light:dark cycles for circadian entrainment, and then may transition to constant conditions for the subsequent 4 weeks. During week 3, mice might receive daily intra-peritoneal injections with a combination of insulin and glucose (or control saline), whilst being fed either ad lib or having food removed for 18h each day - identical to the popular human diet plan known as intermittent fasting. In the following weeks, all four experimental groups would then be returned to constant conditions and then humanely killed at the end of week 5, with tissues being taken for subsequent analysis. This type of experiment would detect

differences in temporal organisation of behaviour, when mice are in receipt of competing timing cues, during week 3, and thereafter during weeks 4 and 5. Other typical interventions might include sleep restriction (by placing interesting novel objects in the cage), or the delivery of drugs via drinking water that modulate the circadian timing mechanism either directly, or by activating a transgene. By monitoring each animal's changing locomotor activity over time we can assess how experimental manipulations have affected their circadian rhythms. On rare occasions, this type of experiment might be combined with prior surgical implantation of wireless telemetry devices that allow changes in body temperature, heart rate or brain activity to be passively monitored in parallel. Animals will be monitored on a very frequent basis as they recover from surgery, with all possible steps being taken to minimse their discomfort. Animals may be genetically modified, for example, they may carry mutations in a "clock gene" that mimics human sleep disorders. In our experience, such genetic modifications manifest with very mild phenotypes in mice, without major adverse effects.

What are the expected impacts and/or adverse effects for the animals during your project?

Surgical mice will experience some discomfort after surgery, and this is expected to last no more than 24 hours. Very rarely after surgery the severity of clinical signs can be such that the humane end points may be reached. Because we are interested in understanding how our internal clockwork functions in the body, in some cases we will use common, non-toxic drugs to interfere with these processes. Unless otherwise specified, the administration of substances and withdrawal of body fluids will be undertaken using a combination of volumes, routes and frequencies that of themselves will result in no more than transient discomfort and no lasting harm. For this reason, we strongly favour the delivery of substances in drinking water or food, wherever possible.

As social animals, social isolation is stressful for mice; however, group housing is more stressful than single-housing for males in the common mouse strain we use (C57/Bl6, Kamakura et al, Physiol Rep, 2016). To assess the circadian behaviour of the animal, mice are singly-housed (to ensure only their own clock controls behaviour, and not physical interaction cues) and their activity cycles monitored by a running-wheel and/or passive infra-red movement detectors. Although housed in separate cages, it is important to emphasise that the animals are not socially isolated: visual, auditory and olfactory cues are freely transmitted between the neighbouring cages (3-10 cm away), the tops of which are metal grids, i.e., not sealed. For mice, olfactory and auditory cues are the major conduits for socialisation and, critically, these are not inhibited during behavioural recordings. Therefore, for experiments where multiple recordings are made at intervals from the same mouse, females are returned to group housing between recordings, whereas males remain singly but socially housed (i.e. they share a large cage with another male, separated by a cage divider).

Typical recordings involve 20 animals per cabinet and always more than one mouse per cabinet. The standard behavioural monitoring and environmental perturbations we employ have a very long history (>50 years) of safe use in mice, with no adverse effects being expected to result from single-housing, transient restriction of sleep, food or changing lighting/temperature conditions, i.e., the potential adverse effects are known but are not observed under the refined conditions that we use.

Depending on experimental requirements, behavioural recordings would usually last for about 5 weeks - the typical minimum necessary to acquire reliable data. Under conceivable but very rare circumstances (i.e., an aging study where repeated behavioural

measurements were required at intervals but could not be performed using female mice only), the total duration of single (but social) housing may be up to 12 months for male mice. Under these conditions, we have not experienced any adverse effects on the animals over >10 years using this procedure: mice retain excellent body condition and levels of locomotor activity. Indeed, in light of their active voluntary use, we view the running wheels as environmental enrichment.

Within the limits of experimental constraints, we regularly provide further environmental enrichment (e.g., sunflower seeds, bedding packs, gnaw bars). Thus, whilst inappropriate housing of animals can constitute a harm, there is no reason to believe that the conditions we use are harmful in the short or long-term because animals are not socially isolated and the frequency with which clinical signs are observed is not greater than under standard housing conditions. Whilst we have never observed evidence for cumulative harm during experiments with several steps that run over several months, we are aware of the possibility and actively monitor for it.

The vast majority of adverse effects encountered by mice undergoing procedures on the PPL are expected to be mild, and few in number. The protocols will involve breeding and generation of genetically altered mice so that we can investigate their biological timing at the level of individual cells, tissues and in whole animals. This project also involves using mice carrying genetic manipulations which we already know or have good reason to expect will be non-harmful, with the exception of P301S T43 tau mutant mice which may start to develop movement the first symptoms around the time that they are due to be culled. The experiments we perform will be designed to ensure that the smallest possible number of animals is used, and that they endure the least possible suffering.

A small number of animals may be subject to the implantation of miniaturised telemetry devices that measure things like brain activity, brain temperature, muscle activity, heart rate, and body temperature. These animals may encounter adverse effects that are moderate in severity due to surgical implantation of the telemetry devices under anaesthesia. Moderate severity would mean that the miceare experiencing some persistent discomfort, and this is something we can monitor by looking for clinical signs of this discomfort such as reduced body weight and not interacting with other mice. At the end of all such experiments, animals will be killed humanely.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severity for the vast majority of the mice (>80%) will be sub-threshold as we don't expect to work with animals carrying debilitating genetic conditions and design experiments to avoid stress.

Roughly 15% and 5% of animals will transiently experience mild or moderate severity, respectively. For example, mice may reach mild severity when drugs that affect circadian timing are delivered by injection. Mice are expected to reach a moderate level of severity exclusively during surgery and during the period immediately following the surgery, which represents <2% of the time spent by the animal in this protocol.

What will happen to animals at the end of this project?

- Killed
- 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?

To be able to better prevent and treat human disease, we need to understand the fundamental mechanisms of the daily biological clock in every human cell, and how it functions in our bodies. The vast majority of our research occurs in cultured cells in vitro without requiring animals. Ultimately though, living animals and their tissues must be used to test the relevance of our research, as the health-relevant, biological end-points we seek to comprehend (circadian rhythms in human physiology and behaviour, sleep and wakefulness) are properties of the intact brain and body and so only occur in living organisms. The adult mouse is the simplest model organism that recapitulates all the essential elements of human circadian physiology, including the gradual deterioration that accompanies aging.

Whilst many individual features of our biological clocks can already, or in the future will, be able to be studied in cultured cells and tissues, this needs to be proven on a case-by case basis. To this end we monitor the scientific literature and explore new alternatives as they come online. Where we are successful in adopting a new alternative for animal tissues, we share our findings with colleagues to encourage replacement in other labs.

Which non-animal alternatives did you consider for use in this project?

Most of our work (>95%) already occurs in cultured mammalian cells and organoids.

At present these platforms are unable to model all aspects of multi-systemic mammalian physiology that we ultimately seek to understand. In some cases, we have used clinical studies in humans instead of mouse and will continue to do so where practical.

Why were they not suitable?

Cultured mammalian cells and organoids are suitable for the majority of our work. Human studies are often unsuitable, either for ethical reasons, or because of the large variance between individuals compared with inbred mice, and challenge of recruiting a sufficient number of healthy volunteers to live alone in constant darkness over several weeks. Mouse models are employed only when there is no practical alternative means of validating key predictions from our in vitro work, which is required in order to meet the appropriate burden of proof within the circadian research field.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 no more than 12500 mice over 5 years. Our animal numbers assume the introduction and/or generation of 2-3 transgenic animals (as occurred during my current PPL), combined with extrapolations of anticipated breeding from our maximum projected requirement of 30-40 animals per week (~4 litters). Estimates for experimental protocols assume 25 procedures per year with average group sizes of n=8. The exact group size for each experiment will be informed by appropriate statistical analyses.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We already perform the vast majority of our experimental work in immortalised mammalian cell lines and stem-cell derived organoids instead of animals. Moreover, the few longitudinal bioluminescent and electrical activity recordings we make from cultured primary cells/tissues ex vivo, require only a small fraction of the animals that would be required to provide the statistical power achieved by in vivo time course assays (where tissues are collected from several animals each time point).

Where there is simply no practical alternative to collecting measurements in vivo, for the types of parametric measurement we employ, we are familiar with the types of variance to be expected and sample sizes required. In general, we find that for group sizes of 8 the distribution approximates a normal distribution and the relative standard error of the mean is about 10%. This allows for effective parametric statistical analyses and provides a consistent benchmark for our experimental design. Our experience is that one- and two-way analyses of variance, factorial or repeated-measures, form robust tests for our experimental results. Wherever possible, studies exploit repeated-measures design to maximise the efficiency of statistical analysis with these relatively small groups.

Should we start to measure different end-points with which we are less familiar we would seek statistical advice to ensure group size and experimental design are adequate for the likely variance to be encountered. Moreover, it is in the nature of the project as it develops in a series of experiments, that experimental groups of one experiment are repeated as control groups in a later experiment. This allows us to develop progressive assessments of the robustness and reproducibility of our responses, consolidating earlier findings. This approach further reduces animal use.

As part of good laboratory practice we write detailed protocols 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). These protocols are then reviewed within my lab, before being shared with relevant members of our Biological Services Group (including NACWO), for comments and suggestions. The protocol is then revised to enable them to be shared and published according to the ARRIVE guidelines.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The genetically altered animals we use are mice. Where suitable lines are not already bred locally, animals will be sourced from other research communities or approved suppliers. Otherwise, we will make the required lines in-house, where an experience base already exists to advise upon production and breeding performance, thereby ensuring the minimum numbers of animals are used in the programme.

Where appropriate, we run pilot studies to explore the parameters of an effect before the final design is decided upon. Whenever a non-standard experimental design will be required, statistical advice will be sought. Whenever possible we make use of ex vivo recordings, this greatly reduces the instances in which we have to perform in vivo recordings, and thereby decreases the number of animals used under these protocols. However, in most of these cases we still rely upon an in vivo element to validate key predictions in a physiological context. In these cases we will adhere to ARRIVE guidelines. Cryopreservation will be used at the earliest and most appropriate opportunity to preserve important lines and remove the necessity to hold stock for extended periods. On occasion we may need to ensure that our protocols are optimised and this may require the re-implantation of un-manipulated oocytes, embryos or blastocysts; if in vitro validation proves insufficient. Cryopreservation of embryos and sperm will be used for long-term storage of genetically altered mouse lines and pedigree lines.

Rederivation will be undertaken should the health status of the animals be compromised in a way that would significantly affect the welfare of the animals or where the experimental results might be altered unduly.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use transgenic mice, the leading vertebrate model to study gene action because of their relative ease of breeding and genetic manipulation. The enormous recent growth in knowledge of the mouse genome and the ability to manipulate it with temporal and spatial refinement are unsurpassed when seeking to examine the role of circadian processes in mammalian biology. Consequently, a wealth of information and tools are available, both locally and more globally, which will greatly benefit this project: the rich diversity of genetically altered mice provides an unrivalled resource for understanding the molecular genetic basis to circadian physiology. Moreover, refinement of procedures is ever increasing due to the continuing development of mouse lines with, for example, tissue-specific and temporally selectively inducible mutations. Inbred strains will ensure consistency of results, and minimise the variations between individuals, thus allowing us to keep the experimental cohorts relatively small, in compliance with the 3Rs (both refinement and reduction). In turn, the results emerging from this project will increase this information, and have the potential to influence scientific progress.

The majority of our in vivo experiments involve passive, non-invasive measurement of

mouse behaviour or sampling of tissues from humanely killed animals, where wild type and transgenic mice are allowed to express their natural endogenous biological rhythms in response to environmental and systemic timing cues (up to mild severity though mostly sub-threshold). There are circumstances where continuous telemetric measurement of one or more biological parameters must be employed over days-months in order that the greatest amount of information can be collected about physiological rhythms from the fewest possible animals. As implantation of these devices requires surgery, this experimental procedure will be applied to very few mice and is classed as moderate. These types of devices have a long history of safe use for EEG, brain temperature, body temperature, and electrocardiography (ECG), with modern innovations that enable radiotelemetry readouts from the same animal over many months without the need for battery change (e.g. long battery lives of up to 1 year, battery charge by induction and/or magnetic actuation of 'on-off' status for intermittent recording and preservation of battery life).

Why can't you use animals that are less sentient?

Many experiments that are required as the ultimate test of a hypothesis cannot be performed in humans for clear ethical reasons, but would be too far from the research question if performed in lower organisms. Therefore, experimental rodents in general, and mice in particular, are the subjects of choice for studying circadian timing as they manifest robust circadian rhythms in behaviour and physiology. Moreover, the genetic details of circadian biology are closely aligned between mice and humans – for example the proteins are interchangeable in cell-based assays. As such, mice are the least sentient animals that can be used for satisfactory tests of mechanisms that facilitate the circadian organisation of behaviour and physiology in mammals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Besides the clear ethical motivation to minimise any welfare costs to any animals undergoing regulated procedures, we take additional care avoid stress or adverse effects in animals undergoing experimental protocols, since this has the potential to interfere with the circadian physiology we seek to understand. To accomplish this, we constantly re-assess our approaches as improved methods become available.
Some of the refinements implemented during the current PPL are listed below:

Experimental refinements: during an experiment, each animal's behavioural trends over the preceding days/weeks are inspected remotely several times/day. In consequence, any abrupt change in an animal's behaviour can quickly be identified and flagged for more thorough visual inspection. Our introduction of passive infrared monitoring, compliments running wheel activity measurements and adds an additional layer of redundancy in case, for example, a running wheel becomes blocked with bedding material over a weekend. Furthermore, between behavioural recordings, mice can now be housed in pairs (with cage dividers) so that males remain singly-housed without being socially-isolated. Also, during the present PPL, additional wireless temperature/humidity sensors were introduced into the behaviour monitoring cabinets to provide redundancy in case the cabinet's built-in monitoring devices developed a problem.

Surgical refinements: strict aseptic technique is used for surgical procedures, with all staff conducting procedures/surgery being well trained, experienced and competent.

Together with our vet we assess and implement the most effective systems for pain

management pre, during and following surgical procedures. During the present PPL, increased use of the anti-pruritic maropitant citrate in the peri- operative period was found to reduce the likelihood of complications.

Post-operative refinements: during post-operative care our animals are hosted in a temperature/humidity regulated cabinets and monitored post-operatively by qualified staff. During the present PPL we began to house mice in pairs (males or females, with cage dividers) so that wound- healing after surgery is not disrupted by physical interactions but animals are not socially-isolated.

Also, by housing animals in larger enclosures we found environmental enrichment with translucent red houses, tilted open running wheels and sunflower seeds (to stimulate foraging behaviour) helped to reduce complications in singly-housed mice following surgery.

Substance delivery refinements: during the present PPL we dramatically increased the delivery of orally available substances via food or drinking water over the more traditional routes of injection or oral gavage, for example, by using the Nombrero for offering food/gel.

Food restriction refinements: food pellets are removed and replaced each day by hand by the same experimentalist so that mice do not need to experience twice daily changes in change and bedding that could constitute a potential stress.

Sleep restriction refinements: Mice are provided novel objects in their cages for a few hours at the beginning of each day so that their natural exploratory behaviour temporarily overrides their sleep drive. They therefore have more than adequate sleep opportunities for the rest of the day. In consequence, the timing of sleep is experimentally shifted but the animal never experiences the adverse effects of sleep deprivation.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow the Home Office Guidance on the Operation of the Animals and NC3Rs guidelines, specifically the ARRIVE guidelines (Kilkenny et al., 2020).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our animal technicians and NIO keeps us constantly informed with advances in the 3Rs, we follow the NC3Rs newsletter and their seminars and demonstrations. We constantly try to refine our techniques and standardise them once these bring tangible improvements.

89. Effects of electromagnetic fields on flatfish 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

Flatfish, Electromagnetic fields, Windfarm, Behaviour, Subsea power cables

Animal types	Life stages
flatfish	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 investigate the effects of Electromagnetic Fields (EMFs) from Marine Renewable Energy Device (MRED) Subsea Power Cables (SPCs) on the behaviour of flatfish.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

There is very little information on the potential impacts of MRED developments on the behaviour of flatfish species, especially EMFs. As commercially and ecologically important bottom-dwelling species, flatfish are likely to encounter SPCs and be exposed to anthropogenic EMFs. It is therefore important to conduct studies to better understand if and how such EMFs may impact flatfish behaviour.

With global commitments for net-zero emissions and requirements for energy security becoming increasingly critical, the renewable energy sector is expanding rapidly. The need

to better understand the environmental impacts of MRED is therefore more important an ever.

As flatfish are commercially important in the North Sea, concern has been raised to the EU by fishing organisations about this lack of information and anecdotal consequences. Mainly that EMFs may create an artificial wall, disrupting the natural migration of fish. One field study shows the possibility of this.

What outputs do you think you will see at the end of this project?

The principal output of this study will be new, groundbreaking information on the behavioural effects of EMFs from SPCs, at strengths measured and modelled around live cables, on the behaviour of flatfish.

This information will be used for fact-based discussions with all users of the sea. It will be published in peer-reviewed academic journals and disseminated to relevant stakeholders to ensure that the results are considered in future studies and Environmental Impact Assessments (EIAs).

Who or what will benefit from these outputs, and how?

Given the infancy of EMF research in the marine environment, any contributions made underpinned by robust scientific research is invaluable for a wide range of stakeholders.

This research in particular, is driven by the need to bring scientific evidence into the discussions related to the expansion of offshore electricity cables. The research aims to provide insight to the validity of the claim by the fishing industry that a moratorium on SPCs should be put in place due to environmental damages. Outputs will therefore benefit these discussions, which will involve transmission system operators (TSOs), wire and cable manufacturers, national electricity grid developers and operators, environmental, climate change, and human rights non-governmental organisations (NGOs), energy think tanks, and fishing communities, organisations, and individual fishers.

As results from this research will show whether EMFs impact the behaviour of flatfish, outputs will benefit those involved in the consenting process and associated EIAs of offshore renewable energy developments and other SPC projects.

The information gained from this study will also contribute to the advancement of mitigation measures and conservation efforts for these commercially and ecologically important species.

How will you look to maximise the outputs of this work?

This work is being funded by the Renewables Grid Initiative (RGI), a European wide collaboration of TSOs, and cable manufacturers. This network of organisations will allow for wide dissemination of the knowledge gained from this work inside and outside the UK.

Results will be published in a peer-reviewed publication, shared at conferences, meetings and working groups, included in company presentations, and shared on social media. The information obtained will also contribute to a Standard Operating Procedure relating to EMF research currently in draft by several European Higher Education Institutes.

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.

Very little is known about the potential impacts of EMFs on flatfish. As bottom-dwelling species, flatfish are likely to encounter buried SPCs and have been observed around windfarms. Adult flatfish also migrate between spawning and feeding grounds and can be found further offshore than juveniles, increasing their changes of encountering SPCs. There is also the possibility that flatfish may use the earth's magnetic field to maintain their heading, and therefore, be affected by EMFs.

Several species of flatfish are commercially important in the North Sea and concerns have been raised by fishermen across Europe and Scandinavia regarding the potential impacts of SPC EMFs on flatfish.

Adult flatfish are most likely to encounter EMFs from SPC and are the life stage caught by commercial fisheries.

Typically, what will be done to an animal used in your project?

Upon receipt of the animals, all individuals will be given a health check (e.g. health conditions such as parasites, weight and length measurements, overall appearance, behavioural changes) and tagged for identification. Animals will then undergo an acclimation period (e.g. one week or longer if required) within their holding tank.

During the experimental period, each animal will be placed in an experimental tank, only part of which will be exposed to an EMF, allowing animals to move away from the EMF if desired. The behaviour of the animal will be observed and recorded, which will allow for detection and identification of potential changes in behaviour.

Each experimental exposure will last between 2-24 hours. Each individual will undergo multiple exposures in order to compare different types of EMFs and to assess sensory adaptation and habituation to anthropogenic EMFs.

What are the expected impacts and/or adverse effects for the animals during your project?

Potential behavioural changes that may occur include avoidance or attraction of the exposed EMF area, or changes in swimming or foraging behaviours. Abnormal behaviours will be categorised as such when compared to "normal" behaviours (determined beforehand from literature and observations in holding tanks). Any changes are expected to be short-term only and no long-lasting effects are expected.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Only mild severity is expected for all animals

What will happen to animals at the end of this project?

- Set free

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

This research aims to establish information on the impacts of EMFs on flatfish behaviour. There is currently a lack of information on basic flatfish behaviour around SPCs and EMFs, which could lead to the prevention of immediate permitting decisions and cable system designs. Therefore, the use of animals is essential to achieve the objectives of this project.

Which non-animal alternatives did you consider for use in this project?

There are no suitable non-animal alternatives for use in this project.

Why were they not suitable?

The complexity of in vivo systems and the behavioural aspect of this study cannot be accurately reproduced in vitro, hence justifying the use of whole living animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Due to the high variability in individual behaviour and the mild behavioural changes expected, the number of animals will be maximised for the timeframe available for the project. It is expected that the project timeline will allow for approximately 100 trials. With three different treatments (control, AC, DC) per individual, around 36 animals could be tested.

A study using the same methodology on sharks used 13 animals, which did not give a desirable effect size. A larger sample size is therefore recommended.

A previous behavioural study with plaice used a sample size of 40 and a published EMF study used 39 skates.

The estimated number above allows for attrition or rejection of animals based on health (or other reasons) or for the release of animals that do not fall within the desired size range.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The norecopa's PREPARE and NC3R's Experimental Design Assistant (EDA) was used to aid in the experimental design. Whilst the complexity of the experiment could not be fully integrated into the online EDA tool, it provides valuable feedback and planning assistance.

G*Power, a free tool to compute statistical power analyses, was used to check suitability of 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?

Learning from previous studies with the same methods and observing animal behaviour prior to experimentation to refine methods. Bootstrapping or hierarchical modelling during analysis will also be considered to improve use of data, if required.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

This project will involve a common non-endangered species of flatfish as model species to reduce impacts on natural local populations and to ensure results are applicable to a wide audience. Likely species are European plaice (Pleuronectes platessa), dab (Limanda limanda), or lemon sole (Microstomus kitt).

All precautions will be taken to minimise suffering when applying procedures (careful handling, procedures conducted by technically qualified people, consultation with flatfish experts and NVS).

All animals will be allowed a period of acclimatisation to experimental set-ups prior to commencement of all trials in order to recover from the short period of handling and transfer to experimental tank.

Experimental tanks will be subjected to similar conditions (temperature, salinity, dissolved oxygen) as holding tanks to avoid inducing unnecessary stress.

All protocols throughout this project are categorised as mild. The EMF strengths utilised replicate those measured around offshore windfarms in the North Sea. These strengths are relatively low. No lasting harm is expected. Plaice have been used in a survival experiment with much higher levels of EMF and no ill effects were reported. The

behavioural thresholds to electrical stimulus (a component of EMFs) for common sole and turbot were magnitudes higher than those that could be created from the EMF levels generated in this study.

Whilst in the experimental set-up, animals will have the option to remain out of the EMF, which is localised to only one portion of the experimental tank.

Why can’t you use animals that are less sentient?

The chosen species and life stage is commercially and ecologically important in the North Sea and understanding how they interact with SPCs is key to their conservation and to achieving productive discussions with the fishing community (an aim for the output of this project).

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All procedures used have mild severity and previous scientific work has established robust and refined protocols. For example, acclimation time will be given, and animals can choose how much time they spend in the EMF exposure. Animal welfare will be monitored before, during and after trials.

All animals will undergo health assessments upon arrival to the facility and will be monitored for a period of several weeks before experimental use. Measures are in place for the animals to display natural behaviour while in holding tanks (e.g. sand bottom). This, combined with daily monitoring of behaviour in holding tanks allows for thorough checks and ensuring high standards of welfare. Animals will undergo minimal handling. All animals will undergo a final health assessment before being released to the wild at the end of the project. All results obtained, both negative and positive, will be published.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Norecopa PREPARE guidelines and resources will be used. PILhs will also conduct literature searches for new research and consult with experts if required.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Regular contact with NIO/HOLC and NACWO who is on NC3Rs and norecopa mailing lists.

90. Neural basis of behaviour in the mouse brain

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Brain, Behaviour, Vision, Cortex, Rodent

Animal types	Life stages
Mice	adult, pregnant, embryo, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We aim to understand how neurons throughout the brain encode signals coming from the senses (such as vision and hearing) and from internal processes (such as engagement, prediction, or experience) to select the appropriate actions.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Life involves a series of decisions. Some of those decisions are based on the senses (e.g., vision or hearing). Others are based on previous experience (e.g., knowing whether a food is good or not).

Many decisions involve multiple factors in combination. To understand how the brain makes these decisions we need to understand how myriad neurons distributed all over the brain encode sensory and non-sensory signals and choose actions accordingly. This is not only important in and of itself but is also a fundamental requirement to enable future scientists to design therapies for diseases such as epilepsy, depression, or schizophrenia, or to develop better sensory prostheses.

What outputs do you think you will see at the end of this project?

Our main outputs are scientific publications and presentations at conferences, for the purpose of enhancing our understanding of the brain.

We also produce software, methods, and devices that are used in neuroscience research to control equipment, acquire data, and analyse data. We then make these freely available to the scientific community, where they are often widely adopted.

Who or what will benefit from these outputs, and how?

Our outputs mainly benefit scientific research and the scientific community. Our research is typically divided into projects. As a project evolves (1-3 years), we present intermediate results at international conferences. We then put the final results in a scientific paper, which we release via a preprint server, and submit to a journal for peer review and publication. Upon publication we make the data freely available, and we provide access to any new software, methods, and devices that we have developed for the study.

How will you look to maximise the outputs of this work?

Our approach is highly collaborative. First, we are part of a wider consortium with multiple laboratories in different countries that share resources, experiments, and publications to tackle complex scientific questions. Second, we are part of a collaboration between scientists and engineers that delivers transformative devices for brain recordings.

We disseminate new knowledge through publications, review articles and conference presentations. We report not only on successful approaches, but also on any limitations that we discover in established techniques. With each publication, we typically also release all relevant software and data. These releases have formed the basis of multiple studies by other groups, confirming that our approach is useful to the neuroscience community, and providing multiple uses for the data we acquire.

Species and numbers of animals expected to be used

- Mice: 10,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mammals share a fundamental brain plan. To study this plan, many neuroscientists have converged on mice due to their rapid breeding and maturation, well-established husbandry and infrastructure, and possibilities for genetic control. These advantages and the vast amount of information that is already acquired about the mouse brain make mice the most efficient species in terms of resources and research impact.

Moreover, in the mouse we have access to brain activity at the level of vast populations of individual neurons, and we have tools to manipulate the underlying circuits.

We study behaviour and learning in adulthood, so we focus on young adults.

However, some of our procedures (e.g., tracer injections) can be more effective if performed earlier in development. In those cases, we perform the procedure in neonate or juvenile mice and then let them reach adulthood before studying their brains and behaviour.

Typically, what will be done to an animal used in your project?

Mice (transgenic or wild type) are bred using standard procedures (Protocol 1). Some mice then receive one or more injections in the brain or systemically to manipulate selected types of neurons, e.g., to make them express some genes or to trace their connections.

Mice then proceed to behavioural / neural measurements (Protocol 2). Most mice undergo a surgery where we implant a small head-post so that we can later restrain them in a stable position for behavioural measurements and neural recordings. In the same surgical session, we typically create a window in the skull to access the brain with ultrasound or optical means, or by inserting miniaturised electrical or optical probes. These probes may be implanted for days or months (chronically) or inserted and retracted within a single recording session (acutely). Often, we also inject tracers or genetic material to label neurons or to express genetically encoded sensors or actuators.

In a few mice, we also temporarily inactivate small brain regions (e.g., retrosplenial cortex, or secondary motor cortex) using optical or chemical means.

In very few mice, we may perform a small and precise cut to separate brain regions that would normally be connected, to study their causal involvement in behaviour or neural activity. For instance, we may perform a callosotomy to cut the nerve fibres connecting the two hemispheres or a transectomy to disrupt communication between specific brain regions like the visual and auditory cortex).

Similarly, in very few mice, a small part of the brain may be removed through incision, to access deeper brain regions for imaging (e.g., aspiration of a small region of cortex to enable the imaging of the hippocampus). These procedures do not have any observable impact in the normal behaviour of the mouse.

Whenever feasible, all surgical procedures are carried out in a single surgical session. Occasionally, however, they need to be split into multiple sessions, e.g., to allow for a virus to express or for the mouse to learn a task.
In very rare cases and only if necessary, we perform minor repair or replacement procedures, which are not painful and are performed under mild anaesthesia. These interventions will be restricted to mice that do not have health complications and where the intervention will reduce the overall harm (e.g. developing infections) and increase the amount of data from the same mouse, reducing the number of animals used to achieve project aims.

Some mice are trained to perform behavioural tasks with fluid rewards. For instance, we may train head-fixed mice to run on a wheel to navigate a virtual corridor, or to turn a steering wheel to indicate the position of a stimulus. A training session typically lasts 1-3 hours and is repeated daily for 2-6 weeks, followed by weeks of steady trained behaviour. To train the mice we control their water intake, supplementing their fluids as needed to ensure they receive a minimum daily amount. This amount is calculated based on the

animal's weight, which is monitored daily and is adjusted to keep the weight within a target percentage of their starting weight. Before, during and/or after this training, we may record or manipulate brain activity.

At the end of a series of experiments typically lasting between 3 weeks and 6 months, the mouse will be killed. Typically, we perfuse the heart and extract the brain for anatomical investigations.

What are the expected impacts and/or adverse effects for the animals during your project?

To ensure the well-being of all mice, we adhere to rigorous protocols aimed at maintaining good health and minimizing pain. Following major procedures, mice undergo a minimum of 7 days of recovery, during which preventative and post-operative pain management is provided. While most mice recover uneventfully, a small fraction may experience transient postoperative weight loss or minor localized bleeding, with rare occurrences of wound complications like reopening or scabbing.

Additionally, transgene induction or neonatal injections may lead to altered neuronal function or rejection by the mother, impacting only a very small percentage of animals. Administration of substances, via tail vein or retro-orbital injections, may rarely result in localized bleeding or other reactions such as swelling or bulging of the eye. Antibiotic administration can potentially cause mild gastrointestinal disturbances such as diarrhoea in a small percentage of animals. After an implantation procedure, some animals may exhibit reduced mobility temporarily, although most adapt swiftly within 24 hours.

Water restriction is generally well-tolerated by mice, resulting in up to a 15% loss of baseline body weight due to reduced food consumption. Mice may also display a dull coat resembling mild piloerection as they physiologically adapt to water restriction (groom less to conserve water). To mitigate potential adverse effects, we monitor their weight and signs of dehydration daily. In cases of dehydration or weight dropping below 85% of baseline, we provide additional water and food treats to bring weight back up to at least 85% of baseline. A small percentage of mice may exhibit altered responsiveness, lethargy, or changes in urine and faecal output. Rarely, mice under water restriction may display territorial behaviour when cohoused.

In a minority of mice, introduction to head fixation may induce temporary distress, evident through vocalization or fidgeting. To minimize this possibility, all mice undergo several handling sessions and gradual habituation to the experimental rig and head fixation.

In a small percentage of mice, interventions to influence neural activity, such as electrode insertion or chemical stimulation, may result in localised bleeding, or stress related to slightly prolonged head fixation.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice: 25% Subthreshold (Protocol 1 only), 25% Mild (Protocol 1 only), 50% Moderate (mice that move from Protocol 1 to Protocol 2)

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Our studies concern the way the brain processes sensory inputs, combines these inputs with internal signals, and uses the result to select and execute actions. To accomplish this goal, we need to study the brains of behaving animals.

Which non-animal alternatives did you consider for use in this project?

We considered the use of human subjects, in vitro assays, and in silico (computer) simulations.

Why were they not suitable?

This work cannot be carried out using in vitro preparations because these preparations are vastly simplified relative to the living brain, and they do not receive sensory inputs, learn from past experiences, and provide motor outputs. Even if they did (it is conceivable that organoids will eventually move in this direction), we would have very little evidence that their function resembles that of the normal brain.

The work cannot be performed in silico because current models of the brain are too simplified to provide insight into the mechanisms of behaviour. Our current knowledge of brain anatomy and physiology greatly limits our ability to replicate the brain in a computer model. Due to the vast amount of unknown information about the brain, these models are currently insufficient in generating new insights about its functioning. We will use computer models to help us interpret animal data and generate testable hypotheses for animal experiments, though these complement rather than replace animal use. Rather, computer models enrich our research through a reciprocal process of experimentation and modelling. For instance, a computer model can replicate mouse behavioural data, allowing us to examine the presence and importance of specific components in the actual brain, thereby validating their role in the observed behaviour.

Finally, we cannot carry out this work on humans because human neuronal activity cannot be measured with sufficient precision or scale. Indeed, human brain activity can only be measured with coarse methods such as MRI or EEG, which do not reveal the activity of individual neurons, or with very limited recordings performed during surgery, which only monitor a limited number of neurons in tissue that will be resected.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot

studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

By estimating the fraction of successful experiments for each study and relating it to the number of mice that would constitute a reliable statistical sample for that study.

We have also conducted literature searches to confirm the typical number of mice necessary to achieve statistically significant results in these types of experiments.

We typically use about 10 mice per group.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Over the past years we have greatly increased the number of neurons recorded per mouse. In each mouse, we can now record from ~1,000 neurons with electrophysiology and ~10,000 neurons with two-
photon imaging in awake, behaving mice. We thus obtain stronger data sets while using fewer mice.

We also developed chronic recording techniques to record from the same mice in multiple experimental settings, again greatly increasing the strength of our observations while reducing the number of mice required.

We have identified reliable genetically altered strains for our work, and we will continuously monitor the literature to identify new strains that could further improve the data that can be collected from animals.

We have consulted the NC3R's Experimental Design Assistant as a tool for planning our experiments.

Where possible, single mice are also used for multiple experiments. For example, where we seek to record and manipulate activity, the same mice can be used first for recording and then for manipulation.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Our lab is at the forefront of developing techniques to record from large populations of neurons in individual mice. This not only reduces the number of mice required for our experiments, but it also produces stronger data. Indeed, recording 10,000 neurons in a single mouse is not only more efficient but also much more powerful than recoding 100 neurons in 100 mice, as it allows us to study how the neurons work together.

We manage the breeding colony in line with the best practice guidelines, paying particular attention to genetic stability and breeding performance. Our in-house database helps us make breeding decisions to maintain a colony that produces only the animals needed for experiments.

We also aim to reduce our reliance on multiple intracranial viral injections or on heterozygous triple- and double- transgenic breeding strategies, which require multiple pairings and have low yield (meaning that they require large numbers of mice). We plan to

replace these strategies as much as possible with injections (typically retro-orbital) of viral vectors such as AAV PHP.eB. These vectors are becoming an increasingly valuable tool for transgene expression in the rodent brain, because of their ability to cross the blood-brain barrier and offer uniform gene expression across large brain areas.

To inject these viruses, protocols from institutions in the United States and Europe also use and recommend only retro-orbital (RO) sinus injections, citing several advantages over tail vein injections. The proximity to the brain facilitates efficient virus transport to the central nervous system, ensuring targeted delivery compared to systemic circulation via the tail vein. Retro-orbital injection minimises the risk of unintended peripheral distribution, bypassing peripheral organs and leading to a significantly higher concentration of the AAV vector in the brain.

Notably, when we attempted to replicate this successful transgene delivery using the same virus batch, same titration, and same dilution, using the tail vein route, we failed to obtain expression. The retro- orbital route is thus the most refined method of delivery.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 both wild-type mice (typically inbred), and genetically altered mice. The genetically altered lines involve harmless mutations that allow us to label specific cells so that we can trace their connections or record and manipulate their activity.

Our research depends on the mice being healthy, cooperative, and engaged in the tasks that we train them to perform, so we have many reasons to avoid any suffering. We always use appropriate anaesthetic and analgesic regimes for pain relief during surgery. Mice are allowed sufficient time to fully recover before progressing to subsequent experiments. Many of our techniques in subsequent experiments are minimally invasive, as they involve imaging through the skull or through a cranial window. The components we insert into the brain are typically extremely small, thin, and light. For example, the recording probes are typically 70 µm wide and 20µm thick, about the thickness of a human hair, Similarly, an optical prism weighs approx. 1.25mg, and optogenetic probes typically weigh 50mg. None of these components can cause any pain, because the brain has no somatosensation.

Over the years, consistent refinement of surgical techniques has led to the development of increasingly durable and lighter cranial implants, extending their viability to six months or beyond (e.g., currently, we can consistently image from the same mice with an optical window for up to six months (> 90% success rate) without encountering obstacles such as regrowth of bone, dura, or scar tissue that could impede the imaging process). As a further refinement, we will be evaluating the skull replacement cap, pioneered by researchers in the US. This novel surgical approach involves a single large craniotomy followed by the implantation of a biocompatible acrylic mesh. This innovation enables recording from

multiple brain regions without the need for performing multiple small craniotomies in multiple surgical sessions.

We have engineered lightweight head-holders (improved lighter titanium which weighs on average 0.8g, an improvement from 1.4g previously used) and the lightest weight chronic implant to date (< 3.4g) so that the mice are able to recover their normal mobility and activity in their home cages within a few hours of receiving these implants.

We progressively acclimatise mice to the behavioural rigs, to mitigate stress related to head fixation. These and other measures work: the mice are cooperative and engaged.

We developed a dedicated software that improves and simplifies the daily management and monitoring of weight and water amounts provided to mice on water control. We have found it to be highly effective, greatly reducing the possibility of human error, because the system reminds licence holders to enter key information and calculates quantities such as water to be administered daily and improving communication and monitoring. Mice are cohoused wherever possible and running wheels have been specially adapted to IVC cages to minimise stress.

To motivate mice to perform tasks, we use water control, which has proven to be highly effective with minimal distress. Mice typically tolerate water control with no adverse effects, and body weight provides a robust measure of health before the mouse develops any signs of dehydration (e.g., hunched posture or piloerection). In this way, we can increase the amount of water for individual mice as necessary to prevent adverse effects. To further motivate the mice to perform the task, and with the goal to increase the amount of water they receive beyond 40 ml/kg/day, we typically add sucrose to water rewards during the task.

Why can’t you use animals that are less sentient?

We want our discoveries to extend as much as possible to humans, and the brain of humans has the same fundamental plan as that of other mammals, but substantial differences with that of other animals. We thus work in a mammal, the mouse.
Our goal of understanding the link between brain activity and behaviour requires wakefulness and typically also behaviour. Our recordings are thus in awake mice, rather than terminally anaesthetised ones.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

As described in earlier sections, we constantly seek to refine our procedures. For instance, we seek to reduce our reliance on triple-transgenic approaches (which can result in unnecessary breeding) and replace them with the injection of systemic viruses. Another example is the skull replacement cap, a novel surgical approach that involves a single craniotomy rather than multiple craniotomies performed in multiple surgical sessions. In addition, we constantly seek to reduce the number of injections and other procedures. For instance, we are increasingly coating the surface of our implants with silk fibroin as means to deliver genetic materials to the surface of the brain, reducing the need for injections.

We monitor the health of our mice daily. To minimise stress, before experiments begin, we gradually habituate the mice to being handled and head-fixed. We pay additional attention to mice that have undergone surgery and may have specific needs, such as moist food to ensure weight maintenance and medication to relieve pain or clean and heal wounds

under NVS advice. Mice under water control are weighed daily and inspected for any signs of dehydration. We increase their water availability or remove them from water control as necessary. To help maintain the weight of water restricted mice within the range, we often supplement the water with sucrose and provide food treats (e.g., forage mix)

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

To ensure best practices for refinement we will follow:
Guidance and publications from the NC3Rs and Laboratory Animal Science Association: (https://nc3rs.org.uk/3rs-resources)
The ARRIVE guidelines: (https://arriveguidelines.org/)

PREPARE guildelines: (https://norecopa.no/PREPARE)

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We routinely engage with ongoing institutional and national 3Rs efforts, including establishment welfare meetings and 3Rs days, interacting with the NC3R's regional manager and the Named Information Officer, and reading the NC3R's newsletter. Our laboratory tends to be on the leading edge of these developments.

91. Neurobiology and treatment of cognitive and affective 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

Neuroscience, Cognition, Emotion, Treatment, Mechanisms

Animal types	Life stages
Mice	adult, juvenile, neonate
Rats	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?

This project will investigate the neurobiology of cognitive and affective disorders, to guide the refinement of rodent models for these disorders and to help identify novel treatment targets. It will use normal rodents and rodent models for mental illness to help evaluate potential medicines, and approaches to improve non-invasive delivery of medicines to 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?

Common mental illnesses like schizophrenia, depression and autism all have debilitating effects on cognition (learning, remembering, reasoning) and affective state (emotion, motivation, sociability). There are major problems with current medicines for these

conditions, as outlined using schizophrenia as an example.

Firstly, existing medications mainly reduce non-cognitive symptoms like hallucinations and delusions, and they only do this in ~70% of patients (with the remaining 30% classed as treatment- resistant). Secondly, a significant proportion of patients who do 'respond' end up discontinuing treatment due to side-effects.

Thirdly, and of greatest concern, there are no medicines that actually delay disease progression, or address the life-limiting cognitive and affective symptoms. These are the focus of this application. The new knowledge that will be generated is really important because developing better medicines needs:

- Better insight into normal biology and its disruption in disease, to identify new molecular targets for potential therapeutics
- Rigorous testing of potential therapeutics in well-validated animal models, to identify promising agents at an early stage
- More understanding of how exposure to different risk factors leads to different subgroups of patients - whose disorders may have subtly different biology and need a more personalised approach to medicine

What outputs do you think you will see at the end of this project?

Some of our research is purely academic. The resulting data will advance knowledge of the scientific community as a whole, via presentations at scientific conferences and articles in scientific journals.

Other aspects of our research involve mutually-beneficial collaborations with the pharmaceutical industry. The ultimate aim of this work is to accelerate drug development and improve patient health.

Benefits by the end of the project should include validation of new molecular targets for potential medicines, and identification of lead compounds that interact with those targets. We hope some of these compounds will become candidates for progression to clinical trials, after undergoing additional preclinical and toxicology testing by industrial partners.

Who or what will benefit from these outputs, and how?

Short-term benefits will be new scientific knowledge (on molecules controlling cognition and emotion, including those that could be targeted by new medicines).

The beneficiaries will be neuroscience and pharmacology researchers from academia and the pharmaceutical industry. They will benefit from the knowledge by learning which are the most promising molecules to prioritise for future research, and also which are less promising so should be de-prioritised. These benefits can be expected during and shortly after the end of the licence period. Mid-term benefits will be to pharmaceutical industry collaborators, within and for five years after the licence period. Benefits to patient health are also expected, but these are a longer-term prospect due to the length of time for a preclinical candidate to reach clinical use, which is in the region of 15 years.

How will you look to maximise the outputs of this work?

Benefits will be maximised by national and international collaborations, presentations at scientific conferences as soon as findings are ready for release, and publications in open-

access scientific journals. We will also seek opportunities to make data and tissues available to other researchers if this can provide benefits not possible in-house, such as through the newly-established regional microbiome network. There is increasing emphasis on public engagement and relevant information will be shared with the public in accessible and engaging formats, via blog articles and science fairs.

Species and numbers of animals expected to be used

- Mice: 240
- Rats: 3060

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

This research will use rats and mice because many aspects of their basic physiology are very similar to that of humans. Some of our work will assess the ability of novel formulations to improve non-invasive delivery of medicines directly from the nose to the brain. This is an attractive strategy for medicines that are digested if taken in tablet form. In mice, regions of the nasal cavity we need to target are closer to the nostrils, so administration is even more simple in this species. It cannot use invertebrates or fish because they do not have relevant nasal structures, or direct the nose-to-brain nerve pathways present in mammals.

Another reason for using rats and mice is that many core aspects of their cognitive and affective behaviour are similar to those in humans. These include regulation by shared brain regions with a similar anatomy, plus the same neurotransmitters and receptors. There are also key parallels between rodents and humans in terms of gut microbiome composition, its ability to influence brain function and behaviour, and the gut-brain communication routes we need to study. These are not present to the same extent in non-mammalian species.

We need to use a range of life stages, from embryonic through to adults. This is because the complex mental illnesses we are researching have their origins in early life (e.g. due to maternal infection or medication use) and adolescence (e.g. social isolation and stress). But symptom onset is typically delayed, such that diagnosis and treatment normally occur in adulthood. We will try to detect early molecular changes during adolescence, and see whether treatment with novel therapeutics at that stage can prevent disease onset. The lifespan of rodents is ideally suited to this work and will provide a wealth of data within the duration of this licence.

Typically, what will be done to an animal used in your project?

Most experiments will involve administration of potential medicines and monitoring of behaviour in tests relevant to cognition and affective state. Initial examination will generally use normal animals.

Potential medicines that show promising activity will progress to evaluation in disease models, created by exposing rats or mice to established environmental risk factors for

mental illness. For example, in some studies we might treat pregnant dams with a viral or bacterial mimetic to replicate the effects of infection on her developing offspring. The symptoms in the dam are relatively mild and only last a few days - we consider them equivalent to an illness that may cause humans to stay off work, but might not trigger a GP visit and definitely wouldn't need hospitalisation. In other cases we might restrict social interaction in adolescent animals to mimic the stress associated with lack of positive relationships during this critical window of brain maturation. This has become especially topical following the COVID-19 pandemic.

Normal animals and those used to model mental illness will typically experience mild transient pain or stress (but no lasting harm) from administration of potential medicines by standard routes (oral, intraperitoneal or subcutaneous) or administration of tiny droplets into the nasal cavities to achieve direct nose-to-brain delivery. In some experiments we will need to perform a short surgical procedure (lasting around one hour) to allow administration of the potential medicine, and we will only do this in rats, not mice. For example, in some cases we need to administer potential medicines directly into a specific brain region to learn more about where they work. We will only do this in normal animals, not disease models. In other cases prolonged administration of the potential medicine is needed to see a therapeutic effect, and it is sometimes kinder to achieve this by implanting a small slow-release device to minimise the need for repeated injections. Under general anaesthetic, we would either make a very small window in the skull to gain access to the brain and implant a tiny probe (less than 1cm long and 0.75mm diameter) that we secure with dental cement before closing the wound or make an incision to implant a small pellet (less than 2% of the animals body weight) under the skin or in the abdominal cavity before closing the wound. These animals may experience some mild to moderate pain after surgery, which will be managed with analgesics (under the supervision of the NVS). In cases where prolonged administration of a potential medicine is needed to see a therapeutic effect, but properties of the substance are not compatible with slow-release devices then animals will need to undergo repeat administration by standard routes. In these cases administration will be limited to a total of 28 occasions, at a rate of up to two occasions on any one day.

Normal animals and those used to model mental illness will undergo behavioural assessment in up to five tests designed to provide insight into different aspects of cognitive function or affective state. Most of the tests are innocuous and assess exploration of different zones of mazes, responses to reward, or social activity on a single day. Other tests assess longer-term learning and memory so involve training over multiple days. They may require minor food restriction so that learning and memory can be determined by willingness to perform learned actions to obtain food rewards (e.g. by visiting specific areas of a maze, digging in different scented or textured media, or pressing specific symbols on a touchscreen). The above tests all add variety to the animals daily lives. A small number of tests are designed to assess response of the animals to stress, because altered stress responses are a key feature of mental illnesses. For these tests animals will be confined in a tube small enough to stop them walking about, but not small enough to squeeze them.

Stress responses will be assessed by measuring changes in body temperature and vocal calling. This will only happen on a single day, and for no longer than two hours. A separate small number of tests are designed to assess memory for negative events, because these types of memory gain abnormal prominence in mental illnesses. For these tests animals will be exposed to very mild foot shocks, similar to the static we experience from car doors in dry weather. This would only happen on on single day and would be limited to a maximum of five shocks, each lasting for one second. None of the stressors

used during behavioural testing induce lasting pain or visible signs of damage. But we will make sure that, of the five tests each animal might experience, no more than one involves food restriction, and no more than one involves aversive conditioning/assessment of stress responses. In cases where rats undergo a surgical procedure to enable administration of potential medicines this limit is reduced, to one test involving food restriction or one test involving aversive conditioning/assessment of stress responses.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals exposed to risk factors for mental illness might show a slight delay in reaching developmental milestones (e.g. eye opening 1-2 days late). They might also be less settled or more vocal when handled, but are physically healthy and active. They have relatively subtle differences in the size of some brain structures (-5%; only apparent via MRI scans) and in the balance of chemical transmitters, receptors and inflammatory markers within the brain (that can only be detected via sensitive post- mortem assays). They also exhibit subtle behavioural changes like impaired memory and social withdrawal, that are most apparent to trained observers using sensitive behavioural tasks and comprehensive data collection. However, it is likely that animals undergo moderate levels of psychosocial stress from risk factor exposure, and we will therefore limit the duration of this. We will design all studies to terminate at or before the age of 20 weeks, which allows long enough for the syndrome to establish and assessment of potential medicines to be completed, whilst also minimising the duration of potential adverse effects.

We expect the potential medicines to have beneficial rather then adverse effects. But it is possible that animals might show some mild side-effects, like sedation. These would only be transient (lasting around one hour) and we hope to minimise them by careful dose selection. It is also possible that animals might show some loss of condition in cases where prolonged administration is needed, or loss of body weight in cases where minor food restriction is needed to motivate performance in behavioural tests. We will keep the duration of potential medicine administration and of food restriction for behavioural testing to the absolute minimum necessary to meet our scientific aims.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice: 32% mild, 68% moderate
Rats: 37% mild, 63% 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 makes use of cell cultures, isolated tissues and existing datasets wherever possible. But the brain is very complex and unfortunately we are still not at a stage where where we can advance understanding of the intricate processes underlying normal brain function and mental illness without well-designed studies in animals. Events within the brain are influenced by distant changes in other parts of the body, and these interactions can only occur in intact animals. For example, there are trillions of microorganisms (bacteria, viruses, fungi) living in the intestine. This 'gut microbiome' is influenced by lifestyle factors and stress, and has a profound impact on the brain and mental health.

This occurs via communication across the 'gut microbiome-brain axis, including effects of gut microbiome metabolites on the brain. New knowledge about the brain and gut microbiome-brain axis is essential to identify new targets for medicine development, and to identify effective novel medicines at an early stage. The use of healthy humans or patients in the first instance is not permitted.

Which non-animal alternatives did you consider for use in this project?

I have considered using *in silico* techniques like computer modelling, and *in vitro* preparations - such as immortalised brain cell cultures that replicate indefinitely without needing any new animal use, and patient-derived stem cells that can be collected from skin biopsies and re-programmed to adopt brain cell-like states. I have also considered *ex vivo* preparations like primary cell cultures and brain slices, although these do need to be obtained from animals.

Why were they not suitable?

Computer modelling techniques are of very limited use, because neither the processes underlying cognitive and affective behaviour, nor the impact of mental illness on these processes are understood in enough detail to produce useful *in silico* models. Likewise our current level of knowledge simply doesn't allow the complex changes that occur in mental illness to be replicated *in vitro* using immortalised cell lines.

Patient-derived stem cells are an intriguing tool to model genetic contributions to mental illness, and we do plan to use them for some separate lines of our research. But they are re-programmed from patients skin cells rather than being obtained from the brain. So they lack all of the accumulated molecular changes caused by exposure of the brain to non-genetic risk factors for mental illness (e.g. social isolation). These changes are central to the research covered by this licence.

Furthermore, any unlikely changes in the patients skin cells that might be caused by exposure to non-genetic risks for mental illness would be 'wiped clean' during the process of transforming them to brain-like cells. So sadly this approach cannot provide any insight into questions that will be addressed by gestational and social models under this licence.

Primary cell cultures or *ex vivo* brain slices can provide some insight into the molecular consequences of risk factors for mental illnesses, but to obtain these we would still need to expose animals to the same risk factors and reach the developmental stages proposed here. However, *ex vivo* research does not allow assessment of complex cognitive or affective behaviours, so the resulting data would be severely limited and not provide anywhere near the same scientific benefits.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Experiments involving normal animals will each use an average of 60 animals, and we plan to perform up to three of these per year in rats but only a total of two in mice.

Experiments involving disease models will normally each use 120 animals, and we plan to perform up to two of these per year in rats but only a total of one in mice. At present, we anticipate that remaining work will all involve rats. Some disease models are based on administration of treatments to a pregnant dam and each of these uses around 24 dams, who are estimated to give birth to an approximate total of 336 pups. We plan to perform a total of two of these. Experiments to develop new, even more refined models for mental illness will normally each use 120 animals, and we plan to perform a total of two of these.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Experiments have been designed using the NC3Rs Experimental Design Assistant to confirm that the planned group sizes are appropriate. The advice of a dedicated statistical consultant has also been sought, free of charge through scientific society membership. This allows us to take into account multiple levels of interaction within our data, to minimise nuisance variables like litter of origin (for gestational interventions) and test session/time of day (in all cases).

For some behavioural tests (e.g. object recognition) we have developed a protocol where every animal receives placebo and each dose of a potential medicine in a cross-over design - akin to that used in some clinical trials. On the first day of testing a similar number of animals receive each of the different treatments/dose levels. After an appropriate washout period (~one week) they receive a different treatment and this continues until they have received them all (typically over 4-5 weeks). This allows each animal to serve as its own control and is regarded as an integral part of the study design to comply with the 3Rs initiative and enhance data quality, rather than reuse. It minimises the impact of natural variation between individuals and allows total animal numbers for a four group study to be reduced from 64 to 12, a saving of 75%. The reduction increases to 80% for a five week study, from 60 to 12.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

A range of tissues will be collected and stored for molecular and biochemical analysis by members of our research group (e.g. of gene and protein expression, including hormones, receptors, cell signalling pathway intermediates). This will help meet the key aims of this licence and also provide preliminary data for future grant applications. Where appropriate, we will make tissues and datasets available for use by current or potential collaborators.

Many of the molecular analyses we perform on stored tissue samples involve use of commercially- available antibodies. These allow us to make proteins visible, and once we can see them we can measure changes in their levels. Recombinant technology means commercial antibody suppliers are increasingly able to provide synthetic antibodies that have not been produced in other animals (like rabbits, goats, sheep and horses). Where suitable recombinant antibodies are available we will always use them instead of products produced in animals.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

This work will use normal rats and mice to provide insight into the brain regions, neurotransmitters and receptors likely to underlie cognitive and affective behaviour in humans. It will also use rats and mice exposed to environmental risk factors for mental illness to help identify potential medicines for human mental illness, and the complex molecular changes produced during disease development.

Rodent models traditionally used for this type of work have been criticised for a lack of predictive validity, which is the term used where effects of a potential medicine in rodent models does not match those in human clinical trials. However, the traditional models have been far too simple. For example, they commonly involve a single injection of recreational stimulant drugs like amphetamine (speed) to trigger an increase in extracellular levels of dopamine within the brain. But this does not impact on other neurotransmitters, inflammatory mediators or the gut microbiome-brain axis, that we now know all show important changes in mental illness. Other traditional models have involved modification of genes like 'disrupted in schizophrenia 1' (DISC1) - but some of the largest genetic association studies have since been unable to link these to human illness.

There is growing acceptance that models like the ones we plan to use are amongst the most refined of those developed so far. This is stated in recently published (2022) scientific literature from internationally-respected researchers outside our group. Models based on relevant environmental risk factors or combinations of risk factors, from gestation through to early adulthood, replicate the complexities of human life experience and produce a spectrum of thanges that appear to closely mimic those in patients. This makes them much more suited to valid evaluation of potential medicines, but does mean the animals need to undergo things like reductions in social contact or environmental enrichment. We will keep the duration of this work to an minimum, and we will never induce pain states in our animals, despite chronic pain also being a risk for illnesses like depression.

Why can’t you use animals that are less sentient?

The comprehensive behavioural tests that we need to perform assess things like learning,

memory, reasoning, problem solving and social recognition. These cannot be performed under terminal anaesthesia or in immature animals. Less sentient animals such as flatworms (Planarians), fruit flies or their larvae (Drosophila melanogaster) and zebrafish only undertake a very limited range of behavioural tests, and these are not currently recognised to have translational relevance to human cognitive or emotional behaviour, nor predictive validity to identify future medicines for human mental illness.

One simple illustration why less sentient species are not suitable for this research involves the hormone oxytocin, which is strongly implicated in social behaviour across mammalian species and is central to some of our research. Instead of having oxytocin, fish/insects/worms posess vasotocin/inotocin/annetocin. These differ from the amino acid building block sequence of mammalian oxytocin by 11-44%, and activate vasotocin/inotocin/annetocin receptors not present in mammals.

This means that potential medicines that interact with mammalian oxytocin receptors are likely to have reduced or no effect in species traditionally considered less sentient than rats or mice.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

In addition to standard daily visual checks by animal care staff, all animals will be formally monitored weekly (by a member of the research group or animal care staff) for weight loss, general condition and demeanour. Animals that undergo anaesthesia and surgery will receive routine pre-emptive and post-operative analgesia plus additional reactive analgesia if required - all under the advice of the NVS or their deputy. These animals will also undergo enhanced monitoring twice daily for four days post- surgery, daily until seven days post-surgery, then twice weekly until the end of the experiment. On each occasion welfare will be scored using facial grimace scales and an AWERB-approved monitoring sheet (that includes characteristics like food and water intake, activity, posture, coat condition, and wound condition). Wet mash will be offered for the first few days following surgery, and at any point where condition of the animal suggests this is appropriate. Any deviations from normal will be documented and discussed with experienced care staff in the Bio Support Unit, including the NVS or their deputy, and appropriate action taken as advised.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use the NC3Rs Experimental Design Assistant and other NC3Rs advice when designing experiments (https://eda.nc3rs.org.uk/experimental-design). We will also refer to the ARRIVE guidelines from the design stage, to ensure that work is conducted to the standards expected by funders and scientific publishers (https://arriveguidelines.org/arrive-guidelines). We will refer to Laboratory Animal Science Association (LASA) publications when planning specific procedures (https://www.lasa.co.uk/current_publications/), and keep up to date with the latest non-animal methods to ensure these are adopted wherever possible (https://frame.org.uk/resources/ and https://caat.jhsph.edu/).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I subscribe to the NC3Rs email alerts, which keep me updated on recent guidance and information. I also receive regular updates and welfare guidance via our Bio Support Unit,

and will actively seek regular refresher training from them (e.g. on animal handling and injections). Methods of best practise are discussed within and beyond my research group via scientific society events and training courses.

92. The immune system in brain health and disease

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Dementia, Brain Health, Immunity, Macrophage, Stem cells

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to understand mechanisms through which brain health is regulated by the immune system.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

This work is important because it will:
Teach us more about how the immune system regulates brain health and disease.
Show us how human genetic change may modulate physiological processes that effect disease processes and tissue health.

Provide novel insights into potential therapeutic interventions in a physiologically-relevant context.

What outputs do you think you will see at the end of this project?

New Understanding: This project will generate a greater understanding of how alterations

in patient genes lead to neurodegenerative conditions via altered cellular function. The cells under study exist in a complex multi-cellular environment and are also studied in the context of chronic disease in which they are implicated. Neither the environment nor the disease process can be replicated in vitro. The protocols on this licence enable the study of cells in a physiologically relevant context permitting a more meaningful understanding of their role in normal tissue biology and disease. The protocols allow the development of mechanistic insights into how genes that are implicated in neurodegenerative disease contribute to disease and also allow for visualisation of the cells in their in vivo context.

Therapeutic Understanding: Ultimately, we are seeking new understanding of mechanisms that underly brain health and disease that can lead to the development of novel and potentially effective therapeutic interventions. One output from this project would be the identification of potentially targetable functional pathways to benefit tissue health.

Knowledge Sharing: This project will generate new information, which will be shared via publication and via availability of large datasets for other to analyse. We hope it will generate new insights into mechanisms that regulate tissue health and disease. We would also hope it would contribute to the development of novel therapeutic approaches and as such may initiate drug development studies.

Who or what will benefit from these outputs, and how?

In the short term (within the life of the project), we would expect benefits primarily to the research community, represented via novel insights and new datasets shared via conventional means (publication and conferences).

In the medium to long term, including after completion of the project, we would hope the models established and characterised would form the basis for physiologically-relevant pre-clinical testing of theories related to the underlying mechanisms of disease and the development of novel therapeutics.

How will you look to maximise the outputs of this work?

Dissemination of research to the scientific community: The data gathered during 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. To accelerate dissemination, we will also use pre-print servers to share our findings prior to scientific peer-review when appropriate.

Dissemination of research to the general public: We prioritise public engagement within our institute and have made our research centre's launch and renewal events public facing rather than purely scientific endeavours. As well as the general public we reach out to patients, carers and other stakeholders and we are establishing a public/patient ‘panel’ to advise and work with our centre. We will continue to hold open days and we regularly invite patients and carers to speak to our research teams to ensure their considerations are in our minds.

Publication of negative data or unsuccessful approaches: A limited number of journals exist that enable to the publication of negative data. Such publication is important to prevent unnecessary duplication of work, but also because technically sound studies with negative results provide meaningful data to be interpreted in the wider context.

Collaboration: Collaboration is key to maximize the quality and breath of data obtained

from an experiment. We have long held collaborations with scientists internationally 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: 17500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 mice is required as valid models of human disease, to examine cells in a physiologically- relevant context, in particular in the brain. We will study mice at all ages, through development, to assess developmental changes, and on through aging to follow the development of pathological processes associated with disease.

Mice are used as the least sentient mammals to model the relevant systems and functions disturbed in human neurodegenerative disease. Mice also provide the most extensively validated models, including with genetic modifications and isogenic controls for addressing physiological, anatomical and cognitive functions.

Most animals will be studied as adults. For example, study of a specific Alzheimer's disease model's pathology from 2 months, which intensifies with age and is associated with increasing pathological markers of disease, but only limited impact on welfare. There are many mouse models of disease and we typically choose models based on their similarity to human disease and/or an aspect of disease and low likelihood of artifacts. Our studies, encompass younger and older mice in recognition of the different stages and processes involved in disease development and sometimes we need to perform treatments on young animals to assess changes in the adult.

We also strive to perform all experiments in both male and female mice. Diseases such as Alzheimer's disease show differential severity and paths with the difference sexes and study of both sexes is essential to understand the underlying disease mechanisms.

Typically, what will be done to an animal used in your project?

In the majority of cases, animals will be bred to obtain specific gene combinations and then the mice will be appropriately to study disease model processes. In these cases, the models used typically have few adverse effects.

In some cases, interventions, such as injection of a substance to examine how cell activity changes in vivo.

In a small number of cases, alternate disease models will be used. In these cases, where there are specific welfare concers, welfare will be monitored carefully and (as appropriate with all protocols) humane end-points will be used to limit severity.

For increased relevance we will also transfer genetically-well defined human cells into mouse models. These models typically require preparation to help transfer of human cells and these preparations will be done in the the neonate. After engraftment, the models may be aged or used in one of the other procedures on the licence.

A minority of animals may undergo appropriate surgical preparation for in vivo brain imaging of cells through a window in the skull.

Mice on these procedures may also be administered by the appropriate route with substances designed to alter or study the immune system. In some cases, substances will be given to induce genetic changes (e.g. drug induced gene changes in transgenic mice).

What are the expected impacts and/or adverse effects for the animals during your project?

Typically the genetic alterations do not result in significant adverse effects except for the immune deficient mice, which have the risks associated with the immune deficiency, such as infectious skin problems (dermatitis, which can be transient) and potentially eye problems. Hence the greatest adverse effects will be due to a surgical intervention, such as required for in vivo brain imaging or establishment of human-to-mouse brain cell chimeras with neonatal intracranial injection. Suitable anaesthetics and pain relief will be used under veterinarian guidance. Typically, animals recover well from these procedures and no overt impact is observed. In some cases, however, animals may experience transient weight loss in the days post-surgery or lethargy. In rare cases the animal may die as a result of the anaesthetic or surgical intervention. However, if this happens, the protocol will be reviewed and, if appropriate, can be modified to reduce the likelihood of this occurring. Any sign of ill effects and the animals are either treated or removed from the study, in discussion with the vet for advice when appropriate. Rarely we will use models of neuroinflammation (variants of experimental autoimmune encephalomyelitis (EAE)) that can be associated with hind limb paralysis. In this latter case, frequent welfare monitoring and the use of humane endpoints limit the severity of disease experienced.

Genetically modified mice will mostly harbour mild mutations (such a fluorescent reporters, Cre recombinase expression, or discrete alterations in specific genes). As such, this mice should not experience any adverse effects. Animals that harbour mutations associated with disease typically have no impairments evident at younger ages, but if kept into older age may start to manifest elements of the disease, which can be specifically monitored for.

This may include features such as weight loss and motor slowing, cognitive deficits, however, the primary models we study (discussed below) do not exhibit these effects, although we will remain alert in aging animals (>12 months).

Administration of substances, typically via injection via suitable routes and regimens, will result in transient pain at the sight of injection.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

At most, the procedures used in this PPL will be classified as ‘moderate’. This will include surgical procedures (e.g. in vivo imaging and chimera generation) and the use of specific

disease models. Administration of substances by needle will typically be classified as mild. The majority of the animals will receive no additional interventions and will be classified as sub-threshold to mild. Immune deficient animals, are susceptible to infection and will be kept in a barrier facility to reduce this, but this can result particularly in skin problems.

Good husbandry, good laboratory practise and discussion with the veterinarian staff when conducting new procedures will help minimise suffering. Where there is consideration that adverse effects are associated with a protocol, the animals will be specifically monitored in a manner appropriate for the procedures.

The majority of the mice used will be genetically-modified, as we use complex genetics to study disease processes and as such the controls often will be required to carry specific genetic alterations to match the background of the test animals. The animals will typically be used according to an equal sex split.

During the course of the project it is estimated that ~80% of animals will undergo no additional procedure; ~10-15% will undergo specific challenges with administered substances; <5% will be used for chimera generation; <2% will be used in stronger challenge models. An overall experienced harms assessment distribution of ~55% subthreshold, ~30% mild, and <15% moderate is anticipated for the project as a whole.

There are no planned procedures authorised as severe and any adverse events that exceed expected severity will be reported under PPL standard condition 18.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

This application aims to understand the mechanisms of, and find new approaches 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 easily or accurately replicated in vitro.

A mammalian species must be used because of the complexity of the immune and neurological systems in mammals. Mice are also the most appropriate species because they are the species with the lowest degree of neurophysioloigcal sensitivity in which genetic manipulation can be reliably and reproducibly achieved and in which many appropriate models have already been made.

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, a functional brain, vasculature and immune system are ultimately required to

assess the functional impact of specific interventions on disease-related processes.

Which non-animal alternatives did you consider for use in this project?

We actively use cell culture to model specific brain cells in a dish (e.g. made from human stem cell lines in culture) or immune cells more generally (e.g. conditionally-immortalized macrophage precursors). These can be genetically-modified in vitro to model specific gene variants and also mixed to generate more complex multi-cellular cultures. Over recent years we have been very active in the generation of novel cell-lines. This has consistently replaced some animal usage under previous project licences. We have published primary research on the validation of these in vitro replacements and to date have generated approximately 80 cell lines and incorporated their use into ongoing studies. We continue to incorporate the production of cell lines into our workflow and future plans, which can also have the advantage of reducing live animal transport as well as replacing animal usage, however, we are increasingly challenged by the importance of tissue context on the validity of our results.

Why were they not suitable?

In in vitro experiments, it is possible to answer simple questions or derive initial data on the impact of specific gene variants or a drug or viral vector on the cells.

However, these systems do not replicate the in vivo complex brain multicellular system, including its vasculature and its interaction with the peripheral immune system, which is increasingly implicated in the neuroinflammatory conditions. This is 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 data from my previous/current PPL to predict future use and calculations associated with anticipated grant applications from my research group.

Previous/current PPL: Our current research is well funded and involves diverse genetic models often bred together to generate mice with 3 or more genetic alterations in defined combinations. This typically requires substantial breeding programmes with ~20 individual lines of animals, including the generation of genetically-modified control mice to match to the experiments. My current license is returning ~3500 mice/year, the majority of which do not undergo additional procedure after breeding.

Our core research is well-funded to 2028 (MRC Programme Grant) and we are applying for new programmes of work (MRC), which will require the generation of further mouse models carrying 3-4 specific genetic variants and these research plans will mean that usage is sustained. We also regularly submit additional funding applications at project grant level.

We use intermittent breeding programme to maintain our lines when appropriate (https://nc3rs.org.uk/3rs-resources/breeding-and-colony-management/worked-example-intermittent- breeding).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

As part of our standard procedures, every experiment involves, prior to commencement, a formal assessment of the design. This including statistical analysis or equivalent where possible of the number to be used. This ensures the correct numbers of animals are used to be able to have a realistic chance to address the scientific question. Typically we use GPower3.1 (Universitat Kiel, Germany) for experimental design assessment. The use of multifactorial designs (both sexes, with and without treatment conditions) increases the power of the experiments as well as providing information both of test conditions and sex variation. Where information is limited with regard to animal variation and effect sizes, we may use the Resource Equation, or we will undertake pilot studies to better delineate the parameters under investigation.

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 appropriate numbers of animals achievable. Sometimes, measuring multiple parameters in the same animals can reveal a parameter with less variation that can be used to established the effect sizes and power of a study. Additionally, we may conduct pilot studies to develop primary data upon which we can design the studies. Additionally, specific ‘refinements’ and careful consideration of procedure can also aid in accuracy and minimising of data variation (e.g. use of fine, low dead space needles and carefully controlled carrier buffers can limit background inflammation in acute inflammatory studies, increasing the signal:noise, and ability to detect differences, reducing the requirement for larger numbers 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.

To model Alzheimer’s disease we primarily use the APP-NL-G-F model. We have found this model to be well tolerated, developing amyloid deposition, gliosis and synapse loss with limited impact on normal behaviour or evidence of suffering. We may also use alternative models with similar life experience, e.g. APP_SAA. Similarly for Parkinson’s disease, the Lrrk2-G2019S model is similarly well tolerated. We breed these models to other mice harbouring discrete alterations in genes of interest (typically those implicated in disease processes or related genes). These gene variants rarely cause any obvious impacts on animal welfare and in many cases mimic the natural variation seen in human

genes. Also we use mice expressing reporter genes, which again do not impact welfare, but simply provide a biological readout for our studies.

For many of our studies, the animals are aged without further manipulation. In some cases they are administered with immunomodulators/immune challenges (including microbes and viruses), biological tracers or similar reagents to test and study the immune response.

The EAE and related models are unique, modelling neuroinflammation, demyelination and synapse loss, this can lead to a moderate level of suffering, but will be monitored closely to limit that suffering.

Why can’t you use animals that are less sentient?

The primary studies in the protocol involve studying disease and immunological processes over a protracted period of time in living animals (with potential intervention from the neonatal period to throughout adulthood). The combination of developed/ing living systems and complex microanatomical architecture and cell:cell interactions in the context of modelling human conditions, with the ability to exploit the experimental genetic-tractability of mice, makes the mouse the least sentient appropriate animal for these studies.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have consistently attempted to refine our procedures over the previous licences in manners which are compatible with the scientific question, for example: i) we have titrated doses on inflammatory agents to use them at the lowest dose; ii) we administer substances by the least adverse method, ie. where injection site is determined we use the smallest gauge needles possible; iii) live infection experiments are also initiated using low-dose challenges with the monitoring of animal welfare compulsory and especially frequent during periods of increased likelihood of adverse effects, to ensure humane evaluation of health and adherence to humane endpoints; iv) we have introduced incentivised pipette feeding (where mice comply) as a refinement over oral gavage, the repeated use of which may cause irritation. We have found incentivised pipette feeding effective and shared this approach with colleagues, where this is not appropriate, we can include rest days in the gavage regimen; v) When injecting neonates they must be separated from their mother. A light anaesthesia of the mother can help with return of the pups, which are rubbed in nesting material to help prevent rejection. Additional use of midazolam can also help prevent rejection caused by the separation. We will continue this by, for example, exploring the use of implanted minipumps as an alternative to repeated injections. For the study of neurodegeneration, the use of surrogate markers of disease enables experiments to be finished earlier, which ensures that most mice will not experience the potential adverse effects associated with age. Similarly, where a demyelination model is used, the end points ensure that the experiments end before the mice experience bilateral hind limb paralysis and continuous monitoring of the clinical manifestations of disease should ensure that many mice end these protocols prior to more severe adverse effects.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All our experiments involve a formal preplanning stage during which the procedure is assessed as a whole. Most of our work involves only simple interventions, which are well characterised and we derive background information, such as regards drug delivery from the literature. If we are unsure of the dosing and/or timing of an administered substance

we will use low dose low frequency pilot studies, which we will escalate as required to achieve efficacy with low cost. In addition, we use environmental enrichment as standard, ensure good husbandry practises and involve professional services, such as the Named Veterinary Surgeon, in the design of studies where more experience is required.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our institute locally circulates news of 3Rs innovations, receive communications from the NC3Rs and liaise with our NC3Rs regional programme manager. My team has introduced a number of 3Rs innovation over the years relating to carefully controlled dosing leading to reductions in animal numbers and use of small needle sizes, and the use of incentivised pipette feeding as a replacement to repeated oral gavage. We have also developed an electronic system for sharing of animal tissue between research groups to help reduce overall requirements for animals.

93. The control of parasitic nematode development

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Nematode, Development, Chromatin, Small RNAs

Animal types	Life stages
Rats	adult
Rabbits	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of the project is to investigate the epigenetic control of parasitism gene expression in the parasitic nematode *Strongyloides*. Specifically, we will study the role of chromatin domains and small non-coding RNAs (sRNAs) in controlling the expression of genes used specifically in the parasitic phase of the *Strongyloides* life cycle.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Parasitic nematodes cause 4 of the World Health Organization’s 20 Neglected Tropical Diseases, infect 1.5 billion people, and cause more Years Lost Due to Disability then Malaria, TB or HIV/AIDS. Parasitic nematodes switch between free-living transmission phases and within-host parasitic phases, when they turn on a parasitism gene expression programme. Despite decades of research we don’t know how this fundamental switch is brought about. This work will investigate the basic biology of how parasitic nematodes turn on their parasitism gene expression programme. By understanding this, in the future we will learn how to turn off parasites’ parasitism programmes, thus developing a whole new approach to reduce the burden of nematode infection in vulnerable human populations.

What outputs do you think you will see at the end of this project?

The principal outputs of this work will be new knowledge that we will discover, which we will present in publications and by the deposition of our data in free-to-access data repositories.

Who or what will benefit from these outputs, and how?

In the short-term these outputs will benefit (i) the academic science community by the new knowledge that we will discover and (ii) those seeking to develop new ways to control parasitic nematodes. If in the longer term the knowledge that we discover leads to the development of new ways to control parasitic nematodes then our work will benefit (i) the very many people infected with parasitic nematodes, which would improve their health and wellbeing, and (ii) livestock infected with parasitic nematodes, which would improve their health, wellbeing and production value.

How will you look to maximise the outputs of this work?

We will seek to maximise the outputs of this work by disseminating the new knowledge that we will discover by presenting our work at scientific meetings and by publication. We will particularly aim to present our results to those whose expertise and interest is in developing new ways to control parasitic nematodes, and in so doing this will maximise the chance that the results of our work are translated.

Species and numbers of animals expected to be used

- Rats: 1650
- Rabbits: 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.

We are using adult animals to maintain the parasitic nematode Strongyloides. Strongyloides is an obligate gut parasitic nematode and it cannot be grown in vitro. We need to maintain Strongyloides in animals to generate material for laboratory analyses to study how protein molecules (called chromatin) that are bound to DNA and RNA molecules control Strongyloides' parasitism gene expression. We will focus on the species Strongyloides ratti (which is a natural parasite of rats) but we will also study other species that have different genomic arrangements of parasitism genes. We will use rats for work with S. ratti and another species that naturally infects rats, S. venezuelensis. We will use rabbits for work with S. papillosus, which is naturally a parasite of sheep, but rabbits can be infected. In a section of this work we will use drugs (called histone deacetylase inhibitors) that will change the Strongyloides chromatin proteins and so change its parasitism gene expression programme. Here we will administer the drugs to the host animals, to affect Strongyloides inside the host.

Typically, what will be done to an animal used in your project?

Typically, an animal will be given an injection to infect it with the parasitic nematode *Strongyloides*. Rats make immune responses against the infection so that they lose the infection after about 4 weeks and cannot be re-infected, so new, *Strongyloides*-naive rats, need to be infected. Rabbits maintain their *Strongyloides* infections longer and to maintain the infection secondary infections may also be given to rabbits.

While animals are infected we will collect faeces from them to prepare larval stages of *Strongyloides* for laboratory analyses. We will also kill some infected animals so that we can collect the parasitic stages from the animals' guts, and we will use these parasitic stages for laboratory analyses. We will withdraw food from rats before we kill them, so that their guts contain less digesta, which helps in collecting the worms for later laboratory analyses. Some infected animals will also be administered with HDACi drugs that will change *Strongyloides'* gene expression.

What are the expected impacts and/or adverse effects for the animals during your project?

The animals will experience a brief period of discomfort when they are given an injection to infect them with *Strongyloides*. Animals will be infected with a sub-clinical number of nematodes so that they will not experience any overt harm or distress when they are infected. Infected rats naturally become immune to the infection. The withdrawal of food will cause hunger and distress to the animals.

Administration of HDACi drugs will cause a brief period of discomfort to the animals.

The HDACi drugs that we will use have previously been used in mammals (including clinically in humans) and they are not harmful when used at appropriate doses.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild for all animals

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We need to use animals to study *Strongyloides* because it is an obligate gut nematode of animals.

Strongylodies cannot be grown *in vitro* so it needs to be maintained in animals.

Which non-animal alternatives did you consider for use in this project?

There are no non-animal alternatives to maintaining *Strongyloides* nematodes.

Why were they not suitable?

There are no non-animal alternatives because *Strongyloides* is an obligate nematode that can only to be maintained in animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated the number of animals that will be used based on (i) the amount of *Strongyloides* material that can be obtained from each infected animal and the amount of material that we need for our laboratory analysis, where we have minimised this amount, and (ii) the need to maintain *Strongyloides* infections in new animals because infected animals lose their *Strongyloides* infection and cannot be re-infected, so new, *Strongyloides*-naïve, animals need to be infected.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have estimated the number of animals that will be used based on (i) the amount of *Strongyloides* material that can be obtained from each infected animal and the amount of material that we need for our laboratory analyses, where we have minimised this amount, and (ii) the need to maintain *Strongyloides* infections in new animals because infected animals lose their *Strongyloides* and cannot be re-infected, and so new, *Strongyloides*-naïve, animals need to be infected.

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 animals to generate sufficient *Strongyloides* material that we require for our laboratory analyses.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm

to the animals.

We will infect animals with sub-clinical numbers of *Strongyloides* nematodes such that the animals do not suffer any obvious harm by being infected.

Why can’t you use animals that are less sentient?

The parasitic nematode *Strongyloides* is an obligate gut parasite of vertebrates that is only able to develop in certain species of hosts, and so to maintain it we need to infect the appropriate host species.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will infect animals with sub-clinical numbers of nematodes so that the potential for harm to the animals is minimised.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the NC3Rs website and the peer-reviewed scientific literature for best practice information.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay informed about 3Rs advances by reading NC3Rs publications, by 3Rs best practice information that is provided within our institution, and by continued discussion of 3Rs principles and their application in our regular research group meetings.

94. Understanding the role of inflammation and fibrosis in conjunctival scarring

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Ocular surface, Scarring, Autoimmunity, Therapy, 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?

There is evidence to suggest that by controlling scarring, vision loss associated with ocular mucous membrane pemphigoid (OcMMP, a rare autoimmune disorder affecting the eyes) can be reduced. The aim of this PPL is to understand how this works, and identify new anti-scarring drugs that can be used to alleviate sight loss in OcMMP.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Conjunctival scarring results from many diseases. Among these, a rare autoimmune disorder called Ocular Mucous Membrane Pemphigoid (OcMMP) can lead to irreversible sight-loss. OcMMP occurs in over 20 million people worldwide, and the disease creates chronic conjunctival inflammation, progressive scarring and debilitating symptoms of constant irritation, pain, and dryness (PMID: 25043452, PMID: 34447757). Treatments

involve immunosuppression but these have little effect on scarring. For half of patients, scar formation continues; 20% become irreversibly blind. Therefore, developing new anti-scarring therapeutics may offer innovative solutions to fight against this devastating visual condition.

What outputs do you think you will see at the end of this project?

Based on promising preliminary data, we want to further understand whether anti-scarring drugs can help alleviate conjunctival damage and sight loss in OcMMP. Therefore, by the end of the project we expect to:
Understand how conjunctival fibrosis and inflammation contributes to scarring in OcMMP.

Develop and evaluate the potential of anti-scarring drugs (known and experimental) to treat conjunctival scarring in animal models.

Inform the scientific community and the general public (e.g. OcMMP patients) about the potential of these experimental therapies, through patient and public events, conferences and high impact peer reviewed publications.

Therefore, the information gained from this project may lead to the development of potential therapies to treat OcMMP, but also in a plethora of eye conditions that are underpinned by conjunctival scarring.

Who or what will benefit from these outputs, and how?

This project will enhance our basic understanding to Ocular Mucous Membrane Pemphigoid (OcMMP) and how its progression is influenced by the conjunctival fibrosis and inflammation that leads to scarring in this condition. We aim to alleviate OcMMP by targeting the molecular fibrotic and/or inflammatory pathways leading to conjunctival scarring, so demonstrating the preclinical efficacy of our therapies is a key step to enhance their implementation in the clinic. Therefore, this research has the potential to significantly reduce the clinical, economic and patient burdens associated with OcMMP. In the UK, around 20% of people who develop OcMMP become irreversibly blind, which poses a significant emotional and physical burden for patients.

Short-term (within the five-year project): the beneficiaries will be the local, national and international research communities involved in ocular conjunctival scarring and the molecular pathways underpinning such pathology. Benefits will include scientific discovery, which can be shared through conferences, meetings, publications and social media. Also in the short term, patients will be aware of our work through focus groups on OcMMP, who continually offer invaluable support in finding remedies for this condition.

Mid-term (5-10 years after this project): This project will determine the potential of new therapies to alleviate conjunctival scarring which occurs in OcMMP. Such anti-scarring therapies showed promise in our preliminary studies, including improved visual outcomes. Our multi-disciplinary team (scientists, doctors and pharmacologists) has established collaborations to help bring the treatment to OcMMP patients. In addition, we will actively seek partners who are currently developing drugs with a similar mechanism for application in other diseases, thus accelerating the path to implement our technology towards essential phase-III trials.

Long-term (10 years after this project): Through knowledge acquisition and therapeutic discovery, patients and clinicians will be the most likely beneficiaries to this research. For

OcMMP patients, it will be the development of a treatment which alleviates conjunctival scarring and sight loss, administered in a way that is patient acceptable (topically) and improve current standard of care. For clinicians, the availability of new therapies able to alleviate OcMMP and to a greater extent conjunctival scarring diseases, would involve a revolutionary approach.

How will you look to maximise the outputs of this work?

Knowledge sharing of positive and negative results and approaches: results from this project will be important for advancing our knowledge in conditions underpinned by conjunctival scarring (e.g. OcMMP), new disease targets and any new treatments. The results of the models, techniques and data will be published in peer-reviewed journals to reach a wide audience (e.g. ophthalmology, scarring, inflammation). Any negative data will be addressed and described in order to allow others to learn and optimise methods. Social media and dedicated magazines for the general public will also be used to promote the research and its results. We will also maximize outputs nationally and internationally, by fostering collaborations, disseminating new knowledge, and publishing results and methods to inform the scientific community.

Collaborations: We have established a number of collaborations to optimize formulations (e.g., drug solubility), as well as to investigate pharmacodynamics and the molecular targets of our anti-scarring agents. This will enhance the translation of our therapies into conjunctival scarring, for which we account with excellent clinical support with accessibility to OcMMP patient cohorts at the local level.

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.

A mouse model of OcMMP conjunctival scarring has been investigated and routinely used for vision research. Mouse ocular anatomy and functioning is very comparable to the human eye, and therefore is an ideal surrogate to model the human disease. In addition, mice also recapitulate the underlying pathology of conjunctival scarring, including the fibrosis and inflammation that contribute to disease development and progression of OcMMP from adult ages. Therefore, an established adult mouse model of (induced) conjunctival scarring have been chosen for this project.

Typically, what will be done to an animal used in your project?

In order to induce conjunctival scarring, mice are first primed via a single (intraperitoneal) injection of a defined mixture of reagents to make them susceptible for conjunctival scarring. 14-21 days later, conjunctival scarring is elicited using eye drops containing a trigger (Ovalbumin), which will be administered once daily for a maximum of 10 days.

To evaluate the effect of the therapy in relieving fibrosis/inflammation that contributes to

scarring in OcMMP, some animals will also be treated with eye drops containing therapeutic substances for 7-10 days at different frequencies (e.g. 1x, 2x, 3x daily).

During treatment, animals will be monitored, non- invasively for signs of conjunctival scarring, including eyelid swelling, conjunctival redness, mucous production and tearing. At the end of the treatment, mice will be humanely killed and the eyes collected for further laboratory investigations, so we can understand the level of eye protection achieved by our therapy.

What are the expected impacts and/or adverse effects for the animals during your project?

The mouse model used in this research has increased ocular fibrosis and inflammation following eyedrop challenge (Ovalbumin). As with humans, the progression of conjunctival scarring may induce adverse effects in most mice in the visual system, including eyelid swelling, conjunctival redness, mucous production and tearing. Some animals may also experience weight loss. Therefore, mice will be closely monitored and animals will be humanely killed if such adverse side-effects cannot be relieved in the short term.

The reagents under investigation are predicted to reduce conjunctival scarring, and so lessen adverse effects associated with the model, however pilot studies using small numbers of animals will be used if the reagents and/or dosages have not been used in mice previously. Nonetheless, mice will be closely monitored for any weight loss or abnormal behaviour for (unlikely) intolerance to the therapeutic agent and humanely killed in the event that adverse effects are seen.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

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?

Animal use is central to this project because of the complex nature of the pathology that occurs in the eye. Conjunctival scarring during OcMMP involves a complex fibrotic and immune interplay which cannot be faithfully reproduced using tissue culture or organ explants. Therefore a fully functioning living system is needed so that we can model whether new anti-scarring therapies can alleviate the underlying fibrosis/inflammation pathology affecting this condition.

Which non-animal alternatives did you consider for use in this project?

Wherever possible we do seek to use in vitro cell culture models within our laboratory (e.g. conjunctival cells from human). These cellular platforms will be instrumental for understanding their mechanism of action, identify their possible adverse effects, and infer optimal doses for the in vivo studies.

Why were they not suitable?

We want to understand whether known and experimental anti-scarring drugs can help prevent damage to the eye that occurs in OcMMP (using treatments given as eyedrops). Whilst non-animal alternatives will be essential to identify suitable drugs and understand their precise mechanism of action, mice developing conjunctival fibrosis/inflammation will be essential to understand the therapeutic potential of such therapies.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 in this project are based upon 10 years of experience of experimental design in ophthalmic research using mouse models, as well as in published data and advice from national/international collaborators working on same animal model. Most of the experiments have quantitative end points, and my previous experiments and/or published data have demonstrated that we require around 10-12 animals per treatment group in order to develop statistically sound results. Where these data is not available, we will perform pilot studies based on our experience to provide preliminary data for these calculations.

Although we will reduce the variability as much as possible, some mice have inherent differences in the development of ocular complications and, like humans, may present a less pronounced phenotype.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Our primary goal is to monitor the effects of such therapies to conjunctival scarring and its underlying fibrotic/inflammatory pathology. To avoid experimental biases that could jeopardize the validity of the results, animals will be randomly assigned to different experimental groups wherever possible.

Wherever possible, we will only use drugs in vivo where in vitro approaches have indicated that they can successfully suppress conjunctival fibrosis and/or inflammation (using a sound cellular platform).

All experiments will be hypothesis driven with clear end points to help aid the design.

Study designs are checked against ARRIVE2.0 guidelines. The EDA tool will be used to minimise any design error or bias and to ensure that we report the study results comprehensively. We often consult statisticians to help derive the correct powering of the study or to review our experimental designs before the study starts.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Initial studies will be designed based on previous experiments. Given that I have over 10 years' experience in ophthalmic research using mouse models, I am confident in the experimental design for studying conjunctival scarring and the underlying pathologies contributing to its progression (fibrosis/inflammation).

Additionally, we have good support from national and international collaborators who work on the same animal model and regularly share their experience to help further optimize animal numbers if required. All of our therapies will be administered by topical routes (e.g. eyedrops), for which the experimental design protocols are already in place.

In addition, reduction in animal numbers will be achieved through:

We will obtain several readouts in each mouse, using non-invasive techniques to assess conjunctival damage during the length of the treatment. By the end of treatments, we will harvest the eyes post-mortem, to understand if our therapy can alleviate conjunctival scarring. This approach will allow us to obtain as much information as possible from each animal to drastically reduce their numbers.

We will also use molecular techniques from where we can evaluate conjunctival, corneal and retinal health using minimal quantities of tissue. Therefore, eye tissue will be multipurposed for different readouts.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 experiments detailed here will be carried out in a mouse model of conjunctival scarring. This model has been rigorously characterised and is the most suitable for use as a surrogate of human OcMMP, since it recapitulates the molecular fibrotic and inflammatory signaling that lead to conjunctival scarring during the disease process. OcMMP will be induced via eyedrops as the less invasive route to animals. Animals may experience acute mild to moderate adverse effects normally restricted to the ocular surface (e.g., eyelid swelling, conjunctival redness and tearing).

Why can’t you use animals that are less sentient?

Less sentient animals such as fish are not suitable for modelling conjunctival fibrosis and therapeutic eyedrop delivery (for technical reasons), and therefore will not be suitable to answer our scientific questions. In addition, the use of embryonic and terminal only procedures are not possible, given the need to evaluate the potential of our therapies to alleviate conjunctival scarring and the associated fibrosis/inflammation that progressively develops in human OcMMP.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

As scientists we are aware of the LASA guidelines and have a strong sense of responsibility for the welfare and care of animals bred for and used within our work.

The probability of encountering and adverse outcome will be diminished by planning preliminary studies and by putting in place stringent physiological end point criteria and close monitoring:

We opted to deliver drugs by the least invasive manner (as eyedrops), for the period of time essential to assess their efficacy (~10 days).

We will perform a pilot study for any new drugs not previously used in vivo.

Animals will be humanely killed in the event of them reaching their humane endpoints with regard to body weight loss, body condition and eye health beyond the expected eye disease, as judged using a bespoke welfare scoring sheet and non-invasive biomicroscopy eye analyses.

These refinement steps, and those still in progress, will streamline experiments and reduce the use and suffering of animals. To this end, we will continuously review the models to identify any further opportunities to refine the approaches should they become apparent.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The ARRIVE2.0 and PREPARE guidelines will be used to help plan, design and report on the experiments in order to ensure our studies are conducted in the most refined way possible. In addition, our experimental protocols have been developed according to LASA guidelines, to ensure best refined practice is applied to safeguard animal welfare.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will use the NC3Rs website to stay informed of latest developments, together with newsletters from our animal facility. I am pro-actively informed to continuously improve my animal research practice by attending local workshops and engaging in seminars and events given by the NC3Rs (https://nc3rs.org.uk/webinars).

95. Understanding the effects of ageing upon inflammatory-invoked gastrointestinal sensory signalling processes.

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Ageing gut, Visceral pain, Inflammatory bowel disease, Inflammation, Sensory signalling

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?

Inflammation is a major source of gastrointestinal symptoms and is life threatening in chronic conditions such as inflammatory bowel disease (IBD). The gut responds to inflammatory stimuli by developing hypersensitivity in gastrointestinal neurons that gives rise to pain behaviour and this acts as a warning signal to seek medical help.

However, the aged gut appears less sensitive to respond to inflammation and this can have profound effects upon elderly patient morbidity and mortality through a delay in seeking medical help. This study will investigate how inflammatory stimuli applied in-vitro causes changes in gastrointestinal sensitivity and why these changes appear lacking in the aged bowel.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

An important issue facing the UK population is that an increasing human lifespan is

occurring without a comparable increase in ‘healthspan’ and this is having a negative impact on individual patients within society. Ageing is associated with impaired sensory perception that includes a diminished sensory response to inflammatory evoked gastrointestinal injury which can lead to a delay in elderly patients seeking attention for disease diagnosis and medical advice. This in turn has critical economic and societal impacts including increased health care costs, morbidity and mortality.

The aim of this work is to understand more about the sensory changes in the aged gut that will advance healthcare knowledge for the ageing community in the medical profession.

What outputs do you think you will see at the end of this project?

Studies in which the biological effects of ageing are explored are timely and relevant to an ageing population. Outputs from this project will be in the form of knowledge to help tailor management strategies for elderly populations with IBDs: there is a significant subset of IBD patients in ageing populations and as such understanding how their disease and symptoms thereof differ from younger cohorts will help tailor management strategies for them. Further outputs will be in the form of publications describing the results of this project. The basic biology underpinning this project is poorly understood and so a key area where impact will be explored is disseminating new knowledge in the form of manuscripts to journals, and presentations at key meetings. Our previous ageing project gave rise to a key publication, and talks at two international meetings. Tissue from this original project was also used to supply a satellite project that led to significant outputs. This project will also provide a stock of well-defined aged tissues as part of a biobank for researchers to access. We also have internal and external collaborations that will benefit from aged tissue to conduct pilot studies into the effects of age upon physiological processes.

Who or what will benefit from these outputs, and how?

The immediate beneficiaries of this research project are likely to be fundamental researchers and clinical scientists with an interest in understanding the impact of ageing on sensory signalling from the gastrointestinal tract. The science community at large will benefit from the training and mentoring of young and early- stage researchers who will be working on this project throughout its duration. The pharmaceutical industry and healthcare industries are also beneficiaries. Our work is immediately translatable since there is a clinical need to be met, and the pharmaceutical industry will gain valuable insights into the physiology and pharmacology of ageing and its effects upon inflammatory-invoked pain pathways.

The timescales for delivering these benefits will be in some cases immediate, although the very concept of a project to investigate the effects of ageing upon gastrointestinal physiology means that some knowledge will be learnt in towards the end of this study (Year 3).

How will you look to maximise the outputs of this work?

In terms of maximising outputs from this project new knowledge will be disseminated in the form of conference presentations, journal publications, open access MSc/PhD theses and building further collaborations within the clinical departments that we have initiated this project with. Throughout my research career I have had a good track record of publishing work in abstract and manuscript form, which has led to publications in top physiological journals and oral/poster presentations at key Gastroenterology meetings, so maximising outputs here is built into our current research programme. In terms of collaborations, we

are building clinical links, and outputs from this project will provide a base for exploring further research collaborations with these people.

Species and numbers of animals expected to be used

- Mice: 350 over five years

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

This project seeks to determine the mechanisms that underlie and modulate gastrointestinal afferent sensitivity. Since these afferents interact with and are influenced by other cells in the vicinity of the nerve terminals it is not possible to recreate this environment in cell culture. The complex interactions between the immune system and the nervous system necessitates animal studies. Moreover, because of the invasive procedures required to record from these afferents it is not possible to conduct these studies on human volunteers or patients. So we cannot replace the use of animals in this study. Mice are the model organisms for this study since these are a well-established model for investigating gastrointestinal function and have been utilised in successful projects to investigate the ageing gut.

The strain of mouse employed in this study (C57Bl/6J) is well utilised in ageing models and is also the model of mouse used in transgenic (gene knockout) studies, so any novel findings in this project would dovetail with focused studies using knockout strains of mice. Female mice will be used since this will provide the best welfare/ housing conditions as these animals will be kept in small sibling-based colonies in a designated ageing-housed environment. However, small numbers of aged male mice will also be used to act as both age and sex-matched batch controls.

In terms of replicating ageing in humans, although rodents have a shorter life span than humans the shape of the lifespan curve (often considered a measure of health of the organism) is similar between humans and rodents demonstrating a “universality in ageing”. The project will use aged mice at 3 months (young adult), 12 months (middle-aged) and 20 months maximum (old age) for the duration of this work. Young mice will act as controls for the aged data. The 12 months animals will serve an important role in studying ‘ageing’ as opposed to looking at ‘aged’ animals, since studies have demonstrated age-related effects occur across the lifespan of animals.

Typically, what will be done to an animal used in your project?

Female C57Bl/6J mice will be aged at our facilities and this licence will enable the ageing of animals until a maximum of 20 months age. A PPL licence is appropriate for this study since animals will be aged for scientific purposes above 12 months and will need monitoring for age-related morbidity throughout their lifespan. Ageing in-house allows a better control over their ageing in terms of diet and housing (two important aspects of experimental design that need control to get robust data in ageing studies). It will also reduce the stress of transport of aged animals to the research facility. Ageing studies need to sample time points throughout the ageing process to accurately assess ‘ageing’.

Ageing animals in-house will allow the study of time points throughout the ageing process (3 months, 12 months and 20 months) in far more tightly controlled environments than getting sourced animals at specific ages from breeding facilities.

This would give the data greater reliability and allow for better control the environment the animals are kept in, reducing variability, and hence lowering the number of animals used. Once the animals are at the appropriate age they will be humanely killed and tissues used in physiological studies as part of this project and other tissue stored at -80C in a biobank of aged tissue for future and other collaborative projects.

What are the expected impacts and/or adverse effects for the animals during your project?

Severity Index.

This project doesn't involve administering any procedures to the animals, and as such the severity markers of this project involves monitoring age-related adverse effects in rodents. As such protocols need to be put in place to quantify and respond to age -related issues in a welfare responsive fashion. In terms of lifespans the median lifespan of C57Bl/6J animals is 866 d (female) versus 901 days (male) (Yuan et al., 2009), so the oldest animals that this project will use are at approximately 70% of their maximum expected lifespan. As such we do not expect to see any of the adverse effects associated with very old animals. Jackson Labs have produced an ageing resource library (agingmice.org) that lists age-related changes to the physiology, biochemistry, and immunology of these animals and is an important resource to assess the possibility of adverse effects occurring in animals of specific ages.

In terms of ageing these animals we need to be aware of the physiological changes that occur across the ageing process. We need to ensure that the animals are healthy aged mice and as such we have refined our experiments to use animals at a maximum age of 20 months as our 'old' animals to minimise age-related clinical signs.

The incidence of age-related signs.
Aged 3-6 months: young adults and no senescence.

Aged 12 months: middle aged adults. Some biomarkers showing ageing but 100% alive.
Aged 20 months: old-aged adults. Biomarkers of senescence apparent. 90 % are alive.

The aged (20 months) cohort will be susceptible to the effects of ageing whereas the 12 months and 3 months cohorts will be free in terms of serious age-related senescence. Animal weight increases as these animals age. Female C57Bl/6 mice weigh 23.5±1.8 g at 6 m and 32.6±3.4 g at 20 months (Ackerk-Bicknell et al 2008). Consequently, animals will be fed adlib using Teklad 2014 14% protein diet to limit the development of obesity related diseases.

Older animals will have greying coats, thinning hair, but clinical presentations may also be presents include rectal prolapse, alopecia, ocular lesions and palpable masses. These increase in number as the animal ages, although data from the Jackson Labs suggests that animals aged to 20 months will be mostly free of these adverse effects. For example: a study looking at lung adenomas showed that at 20 months there was no incidences of lung adenomas in female C57Bl/6J strains (Berndt et al. 2011). Whilst this does not exclude the presence of other tumours it is reassuring but we are mindful of the

possibilities of these events developing and have put in place a protocol to deal with this. Animals will be checked weekly for age-related adverse effects and a questionnaire will be implemented to determine and quantify frailty for these animals. Frailty indexes have been assessed for use in rodent ageing studies and have shown close correlation to end of life (Toth., 2018) and humane endpoints will be established for animals deemed to be frail and approaching end-of life.

Humane Endpoints.

Animals that appear to have difficulty in eating or drinking will be monitored closely and, if necessary, will be fed a mush of ground pellets and water to aid feeding.

Should these signs persist for longer than 24 h then these animals will be humanely culled using Schedule 1 protocols. Any animal that loses > 15% of its body weight will be humanely killed using Schedule 1 protocols. Animals exhibiting signs of abnormally low body temperature (measured using thermal imaging camera) will be given extra bedding, warm pads and monitored closely for 24 h. If no improvements are observed these animals will be humanely culled. Animals exhibiting lethargy (reluctance to move when stimulated to do so), balance and gait disturbance, will be monitored for 24 h and if no improvement is seen in these animals they will be culled. Animals showing signs of palpable masses or tumours will be humanly killed immediately.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

50% will be used at 3 months, 30% will be used at 20 months, 20 % will be used at 12 months. These will be the upper age limits for the duration of this project. We anticipate that animals will be largely free of age associated adverse effects before 15 m of age, so based on this 30% are at risk of developing moderate suffering throughout this project.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 seeks to determine the mechanisms that underlie and modulate gastrointestinal afferent sensitivity. Since these afferents interact with and are influenced by other cells in the vicinity of the nerve terminals it is not possible to recreate this environment in cell culture. The complex interactions between the immune system and the nervous system necessitates animal studies. Moreover, because of the invasive procedures required to record from these afferents it is not possible to conduct these studies on human volunteers or patients. So, we cannot replace the use of animals in this study.

Which non-animal alternatives did you consider for use in this project?

The complex interactions that contribute to normal digestive function necessitates animal studies or using in-vitro techniques employing tissue derived from animals.

This project will use isolated cells and tissue removed from experimental animals (in-vitro studies) as appropriate to complement the more integrative experiments provided by the whole animal studies. Human tissue is not viable since obtaining healthy aged tissue is not possible and we cannot obtain neuronal cells such as dorsal root ganglion cells or nodose ganglion cells from human patients. However, work on sensory nerve recordings and some organ bath physiology will be relevant to developing parallel studies using human tissue in the future.

Why were they not suitable?

Since we are modelling ageing in the gastrointestinal tract and how inflammatory stimuli impact upon the development of visceral hypersensitivity there was no alternative to modelling this in animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Animal numbers have been calculated using our extensive use of the mouse for this type of experimental work and from our previous use of the aged model.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Every effort has been made to reduce the number of animals used in the study. In vitro methods will be employed to maximise the use of each animal with functional studies and analytical studies being performed using tissue from the same animals.

Several in vitro tissues (DRG, nodose ganglion cells, intestinal segments) can be obtained from each animal using protocols developed from previous ageing projects and shared when needed. A biobank of fresh and frozen tissue from precisely controlled aged animals will be developed as a resource for the local and collaborative research community. These resources can be iteratively revisited to address emergent scientific questions using tools available to us and other investigators. This project has followed NC3Rs design protocols to aid experimental design and utilized experimental design protocols discussed throughout the Home Office PPL course. Whilst the study has used regular power analysis to cover animal usage in terms of the 3Rs we have also had to factor in attrition of older animals, and we have included a 10 % attrition for 20 month animals.

What measures, apart from good experimental design, will you use to optimise the

number of animals you plan to use in your project?

Optimisation of animal usage will be through using multiple tissues per animal for studies and producing a biobank of carefully aged tissue. Furthermore, our facilities incorporate rigorous local measures that will include that each proposed study will undergo a rigorous evaluation process 1) when ordering animals consideration of the 3Rs (including Biobank sharing tissues) 2) a formal study plan is agreed with NACWO and NVS, which includes justification of the optimal number of animals/group.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Experiments will be carried out in-vitro using isolated cell and intestinal tissue derived from C57BL/6 mice since these are the most common species for use in the study of autonomic neural mechanisms controlling gastrointestinal function. We developed an in-vitro mouse model for studying mesenteric afferent sensitivity because this provides opportunities for use with transgenic animals that enable the molecular basis of sensory signal transduction to be explored and we have developed and characterised an in-vitro model of gastrointestinal motility. In addition, there are also well-established mouse models for the study of many different areas of gastrointestinal physiology. It’s a common model for studying GI physiology since the sensory signalling properties of mouse gastrointestinal afferents are conserved in the human gut and ageing has the same effects on both intrinsic and extrinsic neurons in the mouse and human. Tissue will be isolated from mice at 3 time points, 3, 12 and 20 months, corresponding to young adults, middle aged and old aged.

These time points are well validated in terms of ageing procedures. We intend to use female mice and refine the project by focusing on using larger numbers of young animals including age-matched controls. This way the project will avoid ageing too many animals and as such will reduce the number of possible age-related issues associated with ageing. This has been described in the section above in which proportions of animals at specific ages is discussed.

Why can’t you use animals that are less sentient?

The animals used in this project are small rodents (mice) as they are the lowest animals on the evolutionary scale where suitable models of GI inflammatory diseases have been developed. The project will use experimental techniques developed by using rodent tissue, and mice are an excellent model for human visceral sensation since they contain the same afferent fibre profile that are found in humans. This project licence will enable mice to be aged to a maximum of 20 months and aged animal tissue will be used in experiments to study the effects of ageing upon visceral sensory signalling.

These experiments are well characterised and there are many examples of work using

these protocols from our lab, and others, that can provide a benchmark against which to assess our work.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We intend to refine the project by focusing on using larger numbers of young animals including age- matched controls. This way the number of aged animals can be reduced and as such we can reduce the number of possible age-related issues associated with ageing.

In terms of minimising welfare costs to the animals we will develop a system of weekly monitoring of the animals using the details set out above. This will dovetail with the regular daily technical team evaluation of all our stock/experimental animals held in our facility in accordance with local and national rules.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Where appropriate we will follow the NC3Rs format for experimental design (Percie du Sert et al., 2020). We will also reference recent work that details how to ensure adequate husbandry of aged mice (Toth, 2018; Wilkinson et al., 2019).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Through our AWERB and through the NC3Rs website. In addition, we have developed a robust mechanism for disseminating 3R information through our concordat on openness on animal research working group, animal research webpages and 3Rs presentations at our annual research conferences.

96. The neurobiology of the mammalian circadian body clock

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Neural circuits, Gene expression, Sleep and wakefulness, Neurotransmitters

Animal types	Life stages
Mice	adult, pregnant, embryo, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We aim to understand the timing mechanisms of the circadian body clock of mammals at the level of individual cells and how their time signals are distributed by neural circuits across 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?

Our circadian clock co-ordinates the daily rhythms of physiology and behaviour that adapt us to the world. In modern society, factors such as shift-work, environmental lighting, ageing and neurodegeneration disrupt our circadian system, promoting metabolic, neurological and psychiatric illness. Our work aims to identify the key neuronal and molecular mechanisms responsible for the normal function of the circadian system and thereby inform how to mitigate these challenges to society’s health.

What outputs do you think you will see at the end of this project?

We believe that our discovery research will improve our fundamental knowledge of the circadian system by: a) identifying how “clock genes” and the proteins they encode interact within brain cells to define intervals of approximately one day and so make them “clock cells”; b) characterising how such clock cells interact to enhance their intracellular timing mechanisms; c) to reveal how circadian timing cues generated in this way are conveyed through brain circuits. This new information will be disseminated by academic publications and more broadly via public engagement activities.

Additionally, new lines of mice and genetically encoded reagents will be generated for use by others.

Who or what will benefit from these outputs, and how?

Our research will principally benefit our basic understanding of how the circadian clock generates and distributes time cues across the brain and thereby organises our behaviour and physiology to adapt to day and night. While the focus is on basic research, our discoveries might also have clinically relevant repercussions through the identification of novel therapeutic targets, for example, for the treatment of sleep and other clock-related disorders. Within the context of our work, we have generated and will continue to generate genetically altered mice and reagents that will be of use not only to our project but also to the broader scientific community.

How will you look to maximise the outputs of this work?

Findings will be made available to other scientists through publication in open-access journals and presentations at scientific conferences and meetings. Large datasets, e.g., RNA sequencing, will be deposited in open-access repositories for others to use. New mouse lines will be deposited in national centres for other scientists to use. We are also active in local and national public engagement events, engaging with a wide range of people in schools and science festivals.

Species and numbers of animals expected to be used

- Mice: 36,550

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are the experimental species of choice because it is possible to generate and acquire genetically modified strains, which allow the visualization and manipulation of selected neuronal populations. Moreover, the mechanism of the body clock can only be studied in intact neural circuits that include the necessary cell types and neurochemicals needed to define circadian time. Nevertheless, a large part of our work will employ brain tissue slices in culture taken from neonates and occasionally adults. To understand the neural architecture through which circadian signals are transmitted, we also need to use adult mice. Finally, we need to monitor the behavioural control exerted by the circuits, typically as rest/activity cycles monitored by wheel-running and general movement, and so free-moving adult mice are required.

Typically, what will be done to an animal used in your project?

The advantage of using mice for our project is that we can selectively mutate genes of interest. To this aim we shall generate mutant animals by injections, for example, of genetic material in eggs followed by in vitro fertilization. To make sure that the animals born from these procedures do indeed carry the mutant gene, we take a very tiny piece of tissue from the outer ear and test the incorporation of the genetic modification of interest. This causes minimal distress or pain to the mice. For in vitro, tissuebased studies, mice will be killed by a Schedule 1 method (adults and neonates) or decapitation (neonates) and tissue dissected. This mild approach constitutes the bulk of our programme. In addition, some adult animals undergo surgical procedures and for this reason we expect them to show clinical signs of a moderate severity as a result of stereotaxic procedures, e.g., injection of viral vectors or neural tracers. Surgeries last about 1 hour, during which we make small windows (unilateral or bilateral) in the skull to gain access to the brain for injection. We finally seal the wound with appropriate dressing. Mice are expected to recover very quickly from the surgery, typically they are already walking around the recovery cage 15-30 minutes after the surgery. To study the visual system, and how it connects to clock-relevant regions of the brain, we also perform injection in the eye. The capillary we use for the injection is very small, about 2-3 times the size of a hair. The procedure is rapid (< 15 minutes under anaesthesia). Mice recover quickly and normally show no signs of visual impairment. In some studies, we may implant, under anaesthesia, a sub-cutaneous osmotic minipump to deliver pharmacological agents, for example clozapine N-oxide an agonist of genetically encoded pharmacogenetic regulators of cellular activity. Typically, this would be done a few weeks after stereotaxic surgery in order to allow time for the AAV vectors to express the pharmacogenetic regulator. Subject to experimental design, mice may receive a second mini-pump at a different site after the first is expired. The mini-pumps are a standard device and a longstanding method to deliver precise dosages of pharmacological agents in a sustained and refined manner. The surgery is routine and rapid, with the animals recovering quickly. To manage cumulative severity, the maximum life-time experience of an individual animal will be three surgical procedures. A single procedure may be minipump implantation (limited to 2 per lifetime) or single or multiple injections into the brain/ spinal cord in a single session of anaesthesia or injection into the eye. Finally, mice are assessed for their wheel running behaviour as a clock-controlled output, under both lighting cycles (white light paired with dimred) and continuous dim red or white light, with ad libitum food and water. This is a mild procedure that observes spontaneous behaviour. To probe their circadian control mechanisms, mice may be subject to changes in the lighting regime and/or receive systemic (i.p, i.v.) injections of compounds that will alter the activity of cells and pathways of interest. At the end of the experiments, adult mice will be euthanised using a large dose of anaesthetic followed by cardiac perfusion, which preserves the tissue for subsequent analyses. At no point during this procedure is the animal conscious or feeling any pain. Alternatively, for example, where tissues are required for biochemical analysis, mice will be killed rapidly by a standard Schedule 1 method and fresh tissues dissected.

What are the expected impacts and/or adverse effects for the animals during your project?

Surgical mice will experience some discomfort after surgery, and this is expected to last no more than 24 hours. Very rarely after surgery the severity of clinical signs may be such that the humane end points may be reached. Unless otherwise specified, the administration of substances will be undertaken using a combination of volumes, routes and frequencies that of themselves will result in no more than transient discomfort and no lasting harm.

To assess the circadian behaviour of the animal, mice are single-housed (to ensure only their own clock controls behaviour, and not physical interaction cues) and their activity cycles monitored by a running-wheel and/or passive infra-red movement detectors. The animals are, however, not isolated because auditory and olfactory cues are freely available between the cages, the tops of which are metal grids, i.e., they are not sealed. For mice, olfactory and auditory cues are major conduits for socialisation. Typically we have 20 animals per cabinet and always more than one mouse in the cabinet.

Depending on experimental requirements, such recordings can last for up to 6 months (this would be a life-time maximum for a mouse moving between protocols), although typically they are much shorter, with recordings of 5 weeks being the typical minimum necessary for reliable data. We have not experienced any adverse effects on the animals from many decades of using this procedure. They retain excellent body condition and levels of locomotor activity. Indeed, in light of their active voluntary use, we view the running wheels as environmental enrichment. Within the limits of experimental constraints, we regularly provide further environmental enrichment (e.g., sunflower seeds, bedding packs, gnaw bars).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severity for the vast majority of the mice (>90%) will be mild as we do not expect to work with animals carrying debilitating genetic conditions, nor are we modelling disease. Mice are expected to reach moderate level of severity exclusively during surgery and during the period immediately following the surgery. The highest ever experienced severity should be, we anticipate, 6.5% moderate, 0.5% non-recovery, 93.0% mild.

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 role of neural networks in circadian time-keeping can only be studied in the fully developed brain, which contains the relevant cell types, neurochemicals and patterns of connectivity. Furthermore, the dominant behavioural output of the system, the sleep/wake, rest/activity cycle, can only be examined in the intact animal. Hence, we need to work with developed, intact brains and animals, and monitor their behaviour in vivo.

Which non-animal alternatives did you consider for use in this project?

We have collaborated (and will continue to do so) with colleagues who devise computational models of the circadian system, and have on occasion used these to design experiments and predict their outcomes. In developing genetic modifications and reporters of gene and cellular activity, we routinely use cell cultures (human- and animal-derived) to test our reagents before committing to mouse-based work. Moreover, the field of mammalian circadian biology continues to be informed by studies in fruit flies and epidemiological linkage data, the use of which ensures that our animal-based work is of appropriate focus and context.

Why were they not suitable?

The very small size of the brain structures involved and the prolonged (weeks) time-course of studies mitigates against the use of non-invasive methods of monitoring the activity patterns of the human clock system. Moreover, they cannot provide information about individual neurons. The cell culture models do not replicate the neural identities and complex circuitry fundamental to the circadian control of behaviour. With respect to computational models, these are still extraordinarily simple in comparison to the complexity of the brain, and, even though they are a useful adjunct (indeed we collaborate with modellers at several universities), they cannot, themselves, substitute for interventional experiments. Moreover, with the further development of AI, there is the possibility that it will enhance the computational models of other workers and thereby reduce the numbers of animals we require for active 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?

Husbandry. In order to pursue the key objectives of this licence we shall need to make use of genetically modified animals. We estimate that over the course of the years we will need to make use of about 50 distinct lines. These include mostly but not exclusively: population-specific, recombinaseexpressing lines, which will be used to provide genetic access to defined cell types; various versions of reporter lines (gene expression, cellular activity), which will be needed to visualize those neuronal populations; activity modulators that will be crossed to recombinase-expressing lines in order to alter the activity state of the identified cells. We shall also use lines in which circadian clock genes or genes affecting neurotransmission are mutated. The establishment of those colonies (superovulation, rederivation etc.) will require, based on our previous experience, about 1,600 animals.

Breeding and maintenance of the established lines is estimated to require, based on our recent experience, around 22,500 animals for the duration of the licence, which contribute to the 30,000 grand total for the breeding programme. This is taking into account the need of refreshing any given running line about 3 times per year, by out-crossing, and an expected usage of about 30 distinct mouse lines per year. We plan to maintain for any running lines an average of 4 breeding cages plus stock. The older stock will be culled as new animals are weaned. These numbers ensure a smooth work flow for 4 lab members

in order to provide access to the lines also in the case of parallel work with the same lines of multiple members (a situation that will occur frequently for the most commonly used lines, such as reporter lines). Importantly, we have used our annual return of procedures data to inform the estimates of the number of animals that we will need to use for breeding.

Experimental uses. The bulk (~60%) of our experimental use is in the form of brain slice studies, derived from pups (7,500, i.e., ~20% of all animals on the licence) and sometimes adult stock animals. These contribute to the 30,000 grand total for the breeding programme. These are used to monitor and manipulate circadian clock function in a culture dish. Clock mechanisms are explored by genetic and pharmacological manipulations. To complement this, we shall also conduct neuroanatomical analyses in which brains are processed for microscopic imaging. Such animals may carry genomically encoded anatomical reporters, expressed intersectionally by cell-specific recombinase, and/or the animals may be subject to prior surgery to deliver anatomical tracers. The behavioural effects of circadian manipulations (genetic and/or pharmacological) will be monitored in vivo by recording rest/activity patterns. Some animals will undergo surgery to define and manipulate behaviourally relevant neural pathways of interest and/ or deliver compounds via mini-pumps.

In brain slice and in vivo behavioural studies, we routinely employ a cross-over design, whereby each slice or animal receives control and experimental manipulations. The cross-over design permits powerful repeated-measures statistical analyses, which mitigate biological variance and maximises the usability and data output of tissues and individual animals, each acting as its own control. When treating with drugs, we use concentrations that bracket the published EC50 of the compound to ensure a biologically meaningful outcome. By using in-bred strains (C57Bl6 background) genetic variability is minimised. Years of experience has established that the variance of our measures rarely exceeds 10% of the mean and so this informs the group size power calculations. Typically, based on previous work, a brain slice study will employ ~10 slices (mixed sex) derived from at least three independent litters (to ensure the results are representative). The circadian clock system has a remarkably precise and stable phenotype such that even minor perturbations become readily apparent over the course of several cycles.

For neuroanatomical analyses we require at least 6 brains (3 male, 3 female) again from independent litters. Modern neuroanatomical procedures based on brain-clearing and light-sheet microscopy allow for highly sensitive and comprehensive mapping of circuits in silico that reveal commonality and variability of structures with unprecedented resolution. Where this requires stereotaxic injection, we would not employ sham controls because there would be no marker examinable in such animals and so the procedure would be futile. Similarly, where we conduct surgery to deliver drug-activatable constructs for subsequent behavioural analyses, the control condition will be vehicle injection of construct-expressing animals, rather than sham-surgery.

When new genetic or pharmacological manipulations are introduced, we do so iteratively, starting with pilot studies to test efficacy and then increasing group size until variance stabilises. This defines the effective experimental cohort size for the measures being made. Commonly, what starts as an experimental manipulation in one study (e.g., does treatment X shift the clock?) then becomes the control condition in follow-up work (e.g., does treatment Y affect the response to X?) as we seek to clarify underlying mechanisms. In this way, we develop a strong experimental thread that incorporates replication whilst progressively acquiring new knowledge.

Once we have established the circadian behaviour of particular animals in Protocol 6, they may be suitable for more intensive surgically based study. To facilitate this, they will be transferred to Protocol 7, 8 or 9. This will allow us to reduce the overall number of mice used and it will also enhance refinement as we shall be clear that mice subject to surgery are behaviourally suitable subjects.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

With regard to the husbandry requirements, we made use of the data accumulated in our previous licences because there are no major changes in our generic approach. We regularly assess the breeding strategy to balance the number of cages allocated to particular lines with the changing requirements of the experimental programme. Importantly, we also propose a very similar workflow for brain slice work and behavioural studies: the top-level considerations have not changed and we benefit from accumulated experience of the number of animals required. Nevertheless, we do acknowledge that on-line tools such as those provided by the NC3Rs and G-power have improved considerably in recent years, becoming more relevant to bespoke research programmes. We regularly refer to them, often prompted by the AWERB and NIO as new developments occur.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Cryopreservation will be used to preserve important lines and remove the necessity to hold stock for extended periods. Cryopreservation of embryos and sperm will be used for long-term storage of genetically altered mouse lines and pedigree lines with in vivo viability assessed to ensure that lines can be re-established successfully. Rederivation will be undertaken should the health status of the animals be compromised in a way that would significantly affect the welfare of the animals or where the experimental results might be altered unduly.

To enhance the efficiency of our breeding programme, in 2012 we implemented a regime of minimal disturbance of females after they have littered down. This allowed us to increase post-natal pup survival by 33%. This procedure was formally reviewed in 2015 and accepted by AWERB and the HO Inspector. We therefore implement this reduced intervention regime on an animal welfare basis, for efficient breeding and for reduction of numbers of breeding animals.

As noted above, we generally run pilot studies to explore the parameters of an effect before the experimental design is finalised. Similarly, as noted, we make use of ex vivo recordings to reduce the instances in which in vivo studies are required. Commonly, we obtain several slices from one animal thus maximising our experimental capacity. When publishing our work, we follow ARRIVE guidelines as to reporting of our 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 species of choice in most areas of biomedical research, allowing us to use powerful techniques such as transgenic and knock in/knock out animals, which would not be available for other species. For instance, the mouse is the only animal for which strains are currently available that allow us to identify genetically neuronal populations that would be otherwise undistinguishable. This is crucial for the success of our project, as it relies on assigning individual function(s) to identifiable cell populations.

The ex vivo recording of brain slices are an ideal approach to study circadian phenomena using tissue derived from sub-threshold or mild procedures. Moderate severity relates solely to the surgical preparation of animals for neuroanatomical and in vivo analyses. These analyses are necessary to confirm their in vivo relevance and to contextualise the results from ex vivo studies. Surgery cannot be achieved without this moderate severity. It involves the smallest amount of tissue damage to gain access to the eye and brain, and animals are given extensive post-operative care including analgesics. Animals are closely monitored throughout the experiments and any signs of problems post-surgery are immediately dealt with or, if this is not possible, the animal will be culled. All surgical procedures are carried out with the best possible level of asepsis and according to best practice (LMB Animal Usage guidelines, an internal document).

Why can’t you use animals that are less sentient?

The circadian system of lower vertebrates is far more distributed than that of mammals. In birds, reptiles and fish numerous brain regions and local tissues are light-sensitive and contribute to the synchronisation and generation of circadian behaviour. In contrast, the suprachiasmatic nucleus (SCN) of the hypothalamus is the ultimate co-ordinator of circadian rhythms in mammals and its dedicated retinal input is the sole route for entrainment to the light/dark cycle. The use of nonmammalian vertebrates would not therefore inform on the human system. In terms of development, the mouse SCN forms late in foetal life and is autonomously rhythmic soon after birth, although synaptogenesis is not complete until the second week. By using tissue from pups aged 10 days or older we employ the earliest developmental stage appropriate to our goals. By definition, we cannot conduct behavioural studies on anaesthetised animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Every step of our work has been carefully assessed so to minimise welfare costs and it is constantly re-assessed as improved methods become available. Surgery is the only procedure of moderate severity. The relevant steps include post-operative care, in which our animals are hosted in a temperature/ humidity regulated cabinet and monitored post-operatively by qualified staff. For pain management, together with our NACWO and Veterinarian, we assess and implement the most effective systems before, during and following surgical procedures.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow the Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act and NC3Rs guidelines, specifically the ARRIVE guidelines (Kilkenny et al., 2020).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our Biological Services Group and NIO keep us constantly informed with advances in the 3Rs and we follow the NC3Rs newsletter and their seminars and demonstrations. We constantly try to refine our techniques and standardise them once these bring tangible improvements. Best practice is also shared across the mouse-based groups across other Establishments and Researchers.

97. Mechanisms of sensory function, pain and analgesia

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

Pain, Analgesia, Neuropathy, Inflammation, Sensory dysfunction

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, 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 major aim of this project is to elucidate the mechanisms responsible for sensory transduction and processing and how these are altered in conditions of persistent pain and related sensory dysfunctions. The ultimate objective is to elucidate mechanisms that can be targeted to treat these conditions in patients.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

This work is important because the treatment of persistent pain, sensory dysfunctions and comorbid disorders, such as fatigue, sleep disruption and visceral dysfunction, represent a major medical and socio-economic burden. For example, it is estimated that about 20-30% of the UK population suffer from persistent pain and many of these patients are not adequately treated because of the limited efficacy and side effects of current treatments. There is clearly a need for novel treatments and the discovery of these interventions will require a greater understanding of the underlying mechanisms that are responsible for pain and sensory abnormalities.

What outputs do you think you will see at the end of this project?

We anticipate the following outputs:

Identification of mediators and mechanisms responsible for chronic pain and sensory dysfunctions. It is likely that the precise role and involvement of these targets will differ depending on the precise disorder. We therefore require a variety of different models to investigate their contribution. These targets will inform strategies to develop therapies to treat chronic pain and sensory dysfunction.

Identification of shared mechanisms responsible for pain and symptoms of comorbid disorders.

The new information generated will be readily shared via conference presentations, peerreviewed publications, press releases, social media posts and interactions with patients to maximize the impact of the outputs generated.

Who or what will benefit from these outputs, and how?

Immediate use of data
These studies will provide further knowledge of the properties of sensory systems and the mechanisms responsible for chronic pain and associated sensory dysfunctions. Experimental results will be analyzed and used to determine the direction and design of appropriate and efficient follow-up experiments. The results of the studies will be disseminated to the research community at large by presentations at international meetings and publications in peer-reviewed journals.

Opening of new avenues for research
We believe that the data generated from this project will open new avenues of research. Research into and improved understanding of the mechanisms responsible for chronic pain and sensory dysfunctions will lead to the identification of candidate mediators, cellular signals, ion channels, receptors etc that are responsible for the pathophysiology. Each novel target/mechanism identified will stimulate research to test and validate the hypothesis that it has a role in the disorder.

Practical Applications
We believe that the findings and outputs from these studies will have important applications for the development of strategies to treat chronic pain and associated sensory disorders such as fatigue. For example, our previous studies have shown that the chronic pain symptoms in disorders such as fibromyalgia and complex regional pain syndrome can be transferred to animals by administration of patient antibodies. Such findings demonstrate the validity of the animal studies for our research and will facilitate the identification of mechanisms that are responsible for the patients' symptoms. Our aim is for

our work to be translated into treatments for patients, and our findings have led to one clinical study to evaluate the efficacy and safety of one treatment for fibromyalgia.
The knowledge that could arise from the proposed research would benefit many people within the wider pain communities as we will significantly advance our understanding of the pathological mechanisms underlying chronic pain and comorbid conditions.

Socio-Economic
Chronic pain impacts the quality of life over one billion sufferers globally, and about 20-30% (around 10-15 million) of the adults in the UK. In addition to the costs of treatment, chronic pain has a major economic cost due to workdays lost. The 2008 Chief Medical Officer report states that 25% of pain sufferers lose their jobs; 16% of sufferers feel their chronic pain is so bad that they sometimes want to die. It has been estimated that back pain alone costs the economy £12.3 billion per year. The cost of pain from all causes is far higher.

We believe that the work carried out in this project will have a positive impact in helping to reduce the global burden of pain and related disorders.

How will you look to maximise the outputs of this work?

We have several external academic, clinical and industrial collaborators (national and international). Understanding the neurobiology of chronic pain and sensory dysfunction is of relevance to many audiences including academic and industrial researchers, clinicians, patients and the general public. Different forms of communication will reflect the varied nature of the beneficiaries. Project results will be disseminated accordingly such that the importance of this research is described to different audiences in the most meaningful manner. For example, we will write news blogs/newsletters for patient awareness groups and directly discuss our research with patients at our regular PPI meetings thereby ensuring direct communication with those living with the condition.

Results from this work will be presented at national and international scientific meetings with a focus on basic science and translational research. Data will be published in pre-clinical and clinical science journals including those with an interdisciplinary focus. We will be targeting pain discovery and management communities. Social media such as the departmental twitter feed will detail project progress, conference attendance and journal publication ensuring that the outputs of this work will be maximized.

Species and numbers of animals expected to be used

- Mice: 13,450
- Rats: 1,450

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are principally interested in the mechanisms that are responsible for chronic pain and associated sensory dysfunctions in the mature nervous system. Some studies of sensation are possible in humans. However, the mechanistic questions that we wish to

address require more invasive techniques that are not possible, feasible or ethical in humans. *In vitro* techniques are also not sufficiently advanced that they can model the integrated interactions between non-neuronal and neuronal cells in the peripheral and central nervous systems. One of our strategies is reverse translation where we interrogate the mechanisms operating in humans by administration of samples (e.g. cells, sera, antibodies) obtained from humans to animals to determine if this treatment results in the transfer of the patients' symptoms to the animals. In other studies, the use of genetically modified animals is required to elucidate the role of specific molecules in the condition. Thus, we will undertake some of our work in animals. The use of rats and mice in conditions modelling a sensitized or hypersensitive state, is critically important. The neuroanatomy/neurophysiology of adult rats and mice is well understood and there is a comparable central nervous system complexity to the human. Our experiments will therefore use adult animals. We can identify mechanisms that are responsible for sensory dysfunctions and pain in rats and mice, whereas a cell culture system would not model the necessary aspects of sensory perception.

The underlying causes of pain and sensory dysfunction that we are investigating vary. For example, nerve injury or trauma can lead to neuropathic pain and inflammation results in hypersensitivities. Other human painful conditions may be caused by humoral factors (e.g. antibodies). We therefore need to study a range of models to recapitulate the human condition.

Some of these animal models are of short duration and can therefore be studied acutely. For example, the injection of a chemical agent such as formalin evokes pain-like behaviours with a duration of about 30 minutes. The symptoms in other models develop and change over time, as also seen in humans, and so some experiments may last weeks or months. For example, nerve damage or trauma to model the condition of neuropathic pain in humans results in the development of sensory hypersensitivities over several weeks. The prolonged time course of some experiments and the fact that one of the most important outcome measures in our work is behavioural assessment of the animal necessitates the use of recovery protocols.

Typically, what will be done to an animal used in your project?

Typically, our experimental protocols (in terms of what will be done to an animal used in this project) will follow the sequence of steps shown below, once it has been determined that animal work is necessary for the question at hand:

Animals will be housed in individually ventilated cages with a 12h light/12 hour dark light cycle and fed *ad libitum.*

Apply a treatment. In a limited number of experiments this will be a treatment such as transgene induction (with e.g. tamoxifen) or cell ablation with agents such as diphtheria toxin. Animals will subsequently be used to study the effects of other treatments (a-c) in this section.

More typically the treatment will be one of the following: a) a surgical procedure under recovery general anaesthesia, b) induction of inflammation, c) administration of a neuroactive substance or antibody, or d) induction of diabetes. These treatments will induce sensory hypersensitivities.

However, when not specifically tested the animals generally do not display any marked signs of pain or discomfort.

The primary surgical treatments are intended to induce neuropathy in a peripheral nerve by mechanical (e.g. nerve section, partial ligation) trauma. Alternatively, nerve trauma will be induced by local application of neuroactive chemicals (e.g. cytokines, axon transport blockers). Surgical wounds will be closed with sutures. Animals with neuropathy and associated sensory dysfunction will typically be studied over a period of 3-4 weeks as this time is required for the full development of a neuropathic sensory profile. These animals will experience some discomfort after surgery and will be treated postsurgically with analgesic drugs except when this would compromise the study of the resultant hypersensitivities.

Inflammation will be induced by administration of a chemical agent or UV irradiation. Local injection of chemicals such as carrageenan or lipopolysaccharide into the paw will typically be used to induce and study acute somatic inflammation (up to 48 hours), although the effects of acute inflammation of other target tissues may also be studied. Chronic somatic inflammation will typically be induced by local injection of chemical agents such as complete Freund's adjuvant, nerve growth factor or tumour necrosis factor into the paw or knee joint and studied for up to 14 days. Alternatively, inflammation will be induced by UV irradiation of a limited area of skin (e.g. paw) and the animals studies for up to 7 days.

Other animals will be treated with chemical agents to induce inflammation of individual visceral organs (bladder, gastrointestinal tract or uterus) under recovery general anaesthesia. The animals will be subsequently studied for up to 25 days. For procedures that require surgery, surgical wounds will be closed with sutures, and these animals will experience some discomfort that will be treated postsurgically with analgesic drugs except when this would compromise the study of the resultant hypersensitivities.

Sensory hypersensitivities and sensory dysfunction will alternatively be induced by systemic administration of a neuroactive drug (e.g. cancer chemotherapeutic compounds) or antibodies or immune cells (e.g. patient-derived samples). Animals will typically be studied for up to 4 weeks following treatments to allow full development of the sensory profiles.

Some animals express a spontaneous mutation that leads to elevated glucose levels and diabetes. In some other animals, diabetes will be induced by administration of a substance (e.g. streptozotocin). Animals will typically be studied for sensorimotor function up to the age of 20 weeks.

Test the functional outcomes of these treatments in behavioural studies. We use a variety of behavioural tests for pain sensitivity and sensorimotor function. Most of our pain tests use threshold stimuli (e.g. applying light mechanical pressure) and the animal is free to withdraw from the stimulus at any time. We also use assays that capture more natural, spontaneous animal behaviour – e.g. using home-cage behavioural monitoring of group housed animals. Typically, only one test will be performed per day but where more than one test is required it will not be more than three.

In some experiments we will test the analgesic/neuromodulatory effects of treatments on the induced hypersensitive condition (e.g. administration of analgesic drugs). The effects will be measured by comparing the behavioural measures before and after treatment.

Typically, these treatments will be given on a single occasion and the animals followed for 24 hours after the treatment, although in some experiments we will determine the longer-term effects (e.g. 1 week) of single or repeated treatment. Animals will experience mild,

transient pain and no lasting harm from administration of substances by injection using standard routes (intravenous, subcutaneous, intraperitoneal). Where continued administration is required for prolonged periods, animals will be surgically implanted with slow-release devices such as a mini-pump.

Cull the animal through schedule 1 or perfusion for subsequent *ex-vivo* studies of tissues or gene expression/protein assays.

What are the expected impacts and/or adverse effects for the animals during your project?

The purpose of these experiments is to study the mechanisms responsible for long-term, chronic sensory hypersensitivities resulting from a range of treatments that model clinical conditions in humans. The presence of these sensory hypersensitivities will be studied by stimulating the animals with specific stimuli such as a change in temperature or mechanical stimulation. However, when not specifically tested the animals generally do not display any marked overt signs of pain or discomfort.

Treated animals will be monitored for behavioural signs of discomfort such as vocalization to handling, postural changes, subdued behaviour or a lack of social interaction. As the studies will examine mechanisms responsible for long-term persistent hypersensitivities, animals may be kept for weeks (e.g. 4 weeks), but the durations will kept to the minimum required to obtain meaningful results.

Some procedures will require minor surgery, for example to manipulate a peripheral nerve or to implant a device under the skin. The mice are expected to recover quickly and will be treated post-surgically with analgesic drugs except when this would compromise the study of the resultant hypersensitive states.

Some treatments may result in unexpected weight loss. Animals will be monitored for changes in weight and any animal showing a pronounced weight loss (20%) in the absence of any other clinical signs 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)?

Protocol 1: Breeding and maintenance of genetically altered mice 5% Mild, 95% Sub-threshold.
Protocol 2: Breeding and maintenance of genetically diabetic mice (moderate): Mice: 100% Moderate.
Protocol 3: Sensorimotor and compound testing in naïve animal. Mice: 10% Mild, 90% Moderate. Rats: 10% Mild, 90% Moderate.
Protocol 4: Induction of peripheral neuropathy. Mice: 100% Moderate. Rats: 100% Moderate.
Protocol 5: Drug-induced sensory dysfunction. Mice: 25% Mild, 75% Moderate. Rats: 25% Mild, 75% Moderate
Protocol 6: Immune-induced sensory dysfunction. Mice: 25% Mild, 75% Moderate.
Protocol 7: Induction of inflammation. Mice: 10% Mild, 90% Moderate. Rats: 10% Mild, 90% Moderate.
Protocol 8: Sensorimotor testing in diabetic animals. Mice: 100% Moderate.

Protocol 9: Neuronal identification studies. Mice: 25% Mild, 75% Moderate. Rats: 25% Mild, 75% Moderate
The overall severity distribution for the project is:
Mice. 10.6% Subthreshold. 11.1% Mild, 78.3% Moderate, 0% Severe
Rats. 0% Subthreshold, 14.0% Mild, 86.0% Moderate, 0% Severe

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 principally interested in the mechanisms that are responsible for chronic pain and associated sensory dysfunctions. Some studies of sensation are possible in humans. However, the mechanistic questions that we wish to address require more invasive techniques that are not possible, feasible or ethical in humans. *In vitro* techniques are also not sufficiently advanced that they can model the integrated actions of the peripheral and central nervous systems. One of our strategies is reverse translation where we interrogate the mechanisms operating in humans by administration of samples (e.g. sera, antibodies) obtained from humans to animals to determine if this treatment results in the transfer of the patients' symptoms to the animals. This requires the use of animals to model the complex integrated responses. In other studies, the use of genetically modified animals is required to elucidate the role of specific molecules in the condition. Thus, we will undertake some of our work in animals. The use of rats and mice in conditions modelling a sensitized or hypersensitive state, is critically important. The neuroanatomy and neurophysiology of the rat and mouse are well understood and there is a comparable central nervous system complexity to the human. We can identify mechanisms that are responsible for sensory dysfunctions and pain in rats and mice, whereas a cell culture system would not model the necessary aspects of sensory processing and perception.

Which non-animal alternatives did you consider for use in this project?

We work with clinical colleagues to use human models and tissue samples, where possible. We also use *in vitro* methods (e.g. cellular studies) where appropriate to identify molecular and cellular mechanisms that may be responsible for the altered properties associated with painful conditions and sensory dysfunctions. Database mining is also used to gain preliminary information that can inform or replace animal studies.

Why were they not suitable?

While non-animal alternatives will be used where appropriate, they cannot replicate the biological complexity of the intact animal. The functionality of the circuitries that are responsible for chronic pain/sensory dysfunction require the operation of and interaction between the peripheral and central nervous systems. The integrated behavioural responses cannot be investigated *in vitro* and are too complex to investigate other than *in vivo*.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals used will vary from procedure to procedure depending on the degree of variability in the experimental measures, but our extensive experience with these models has shown that group sizes of 6–8 (rats) and 8-12 (mice) are generally appropriate. For those procedures involving surgery it is scientifically more rigorous to include sham operated control animals in an experiment. However, for techniques which are well established and for which we know from experience that there is no sham effect we will not include such animals in every experiment but refer to historical control data. The effect of sham surgery will be reviewed periodically and when a new experimenter is using the technique.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

I am familiar with the NC3Rs Experimental Design Assistant having used it for calculating animal numbers for grant applications and in previous experimental studies. The use of this planning tool means that the numbers of animals being used in the project is at a minimum.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Reverse translation. One of our experimental approaches to interrogate the mechanisms underlying pain and sensory dysfunction that operate in patients is to administer samples (e.g. cells, sera, antibodies) obtained from humans to animals. With this passive transfer approach we can determine if this treatment results in the transfer of the patients' symptoms to the animals. In this way we are directly investigating relevant mechanisms that operate in patients. Where appropriate, *ex-vivo* tissue preparations will be taken from animals after e.g. behavioural analysis to study the properties of the tissue that may be responsible for abnormal function. Tissue samples not used for a specific study, may be collected and stored for future use, or shared with other groups to minimize the number of animals culled for tissue harvesting.

We will further seek to reduce the number of animals studied by careful experimental design, the adoption of sensitive outcome measures with small variation, and the study of only the most relevant time points. Where possible each animal will be used as its own control using a repeated measures design. Where this is not possible groups of animals will be utilized. In all *in vivo* tests the number of animals in each group will be the minimum required to achieve statistically meaningful and robust 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.

Rodents will be used in these studies as their extensive use in biological research has already provided considerable information on pain pathways. Mice are increasingly being used for sensorimotor studies. Genetically-altered mice may be used where appropriate. These are particularly useful for defining the role of a particular target in the inflammatory or pain process, or for providing the means for examining the activity of a particular treatment at a human disease-relevant target protein in the body. Some experiments will be conducted in rats where direct comparison with previously published studies on rats is scientifically required. Rodents are the lowest vertebrate group on which these types of experiment can be conducted, and many well characterised and limited severity models have been developed in these animals. We aim to use the animal models described in this licence to examine specifically the pain and sensory dysfunctions associated with chronic disease in order to understand the underlying mechanisms and to test the effects of novel treatments. The severity of the models will be limited as far as possible by limiting the time for which animals are kept following surgery, inflammation or induction of pain/sensory dysfunction and, where compatible with the aims of the project, by the use of analgesics.

Why can’t you use animals that are less sentient?

The use of animals that are less sentient is not possible as identification of the mechanisms that underlie pain and sensory dysfunctions requires knowledge of the events and activities occurring in a complex integrated neural system with physiological similarity to humans; hence less sentient species are not suitable.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals will be regularly monitored for post-operative recovery in experiments involving surgical procedures. For the establishment of models, suitable post-operative analgesia will be provided in a manner that does not affect the experimental aims. The severity of the models will be reduced by limiting the time for which animals are kept following surgery, inflammation or induction of pain and, where compatible with the aims of the experiment, by the use of analgesia. Where novel treatments/agents are being used, their effects will be carefully monitored to ensure they do not cause any unwanted harm.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will publish all our animal work in accordance with the ARRIVE guidelines and the International Association of Pain (IASP) guidelines for the use of animals in research available at: https://www.iasp-pain.org/resources/guidelines/iasp-guidelines-for-the-use-of-animals-in-research/. We further adhere to the guiding principles for aseptic surgery (https://www.lasa.co.uk/PDF/LASA_Guiding_Principles_Aseptic_Surgery_2010.2.pdf) and the administration of substances (https://doi.org/10.1258/0023677011911345).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I use the NC3Rs Experimental Design Assistant tool and am a regular visitor to their website to access their resources. As a reviewer for journals that adhere to the ARRIVE guidelines, I am constantly reviewing advances in the 3Rs. I monitor the current research literature for developments such as modified protocols or new experimental approaches that can be used to refine our animal models.

98. The genetic and developmental basis of morphological and behavioural variation in cichlid fishes

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

evolution, organismal trait diversity, genetics, development, cichlid fishes

Animal types	Life stages
Cichlid fishes	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?

To understand the genetic and developmental mechanisms underlying organismal diversification (how organisms evolve and become different species) using the morphological (physical characteristics) and behavioural variation present in cichlid fishes as a model system.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

One of the enduring challenges in evolutionary biology is understanding how organisms adapt and diversify in their surrounding environments. This issue has been recognised as a significant scientific challenge of the 21st century, particularly in light of ongoing environmental changes. Yet, we do not fully understand how new species form and how organisms can rapidly adapt to and cope with novel challenges.

To understand the mechanisms by which organisms diversify and adapt, we need to study which genetic and developmental changes encode adaptation to novel environmental variables. For example, changes in the light environment (e.g., water turbidity) might affect colour attributes and how animals see each other; or changes in food availability, might affect feeding ecology and craniofacial attributes (variable mouth morphologies). So what are the genetic and developmental mechanisms that make just rapid morphological changes possible?

In this project, we study cichlid fishes, a group of animals known for their rapid speciation, diversification and adaptation patterns. These fishes show a lot of diversity in pigmentation patterns and feeding morphologies that evolved as a response to changes in their surrounding environments (such as variable light conditions and novel feeding grounds). Here, we will identify which genetic mutations and developmental mechanisms encode such rapid adaptations. Understanding the genetic underpinnings of adaptation is important so we can understand the patterns of rapid adaptation which will help predict how organisms cope with environmental challenges.

What outputs do you think you will see at the end of this project?

The proposed research project addresses fundamental questions regarding the genomic and developmental basis of adaptation and organismal diversification and also in vertebrate evolutionary biology, therefore the main benefits will be to fundamental scientific knowledge and will generate several publications reviewed by other scientists. We list the main expected findings below:

Identification of genes underlying morphological and behavioural variation (such as variation in head shape, colouration patterns and courtship behaviours).
Identification of cells and developmental process (e.g., pigment cell migration) that generate the variable adult pigmentation and craniofacial morphologies.
Determination of how certain hormones (such as thyroid and sex steroid hormones) control the development of morphological traits and whether these have an impact on the courtship and breeding behaviour of these fishes.

Taken together, these three major findings will uncover fundamental insights into the genetic and developmental basis of rapid morphological change as a response to adaptation to the surrounding environment. For example; what are the type of genetic mutations that encode adaptation - do these mutation involve gene regulation or gene function; are there many genetic/developmental changes required for morphological changes to occur, or does adaptation proceed through a small number of mutations which results in drastic changes in developmental processes with a large morphological effect; how sensible are these genetic and developmental systems to hormonal manipulation which can be easily disrupted by environmental variables?; etc. The answers to these questions will uncover mechanisms of adaptation and diversification and help predict how rapidly organisms respond to changes to their surrouding environments.
Furthermore, given the focus on embryonic developmental mechanisms in vertebrates (e.g. pigmentation, lateral line and craniofacial development), this work will also contribute toward the understanding of how vertebrate organisms function and develop with important implications to all vertebrates including humans.

Data generated will be useful to other researchers and will lead to more collaborative work. We will publish in open access journals and deposit our data in publicly available, free repositories, that can be re-used by other researchers (reduction measure). We will publish both scientific findings as well as protocol development and optimisation.

Who or what will benefit from these outputs, and how?

Short term benefits are further protocol optimisations, improvement of rearing practices and sharing of tools with the community. We are contributing to the development of cichlids as an emerging model system that can be used not only for evolutionary biology but also for developmental biology studies (see our recent review about the potential of the model system: (https://doi.org/10.1186/s13227-02200205-5).
Long term, this will generate knowledge on how organisms adapt and how they diversify and become different species as a response to factors in their surrounding environment. More specifically, we will uncover the genomic and developmental basis of these processes which will help predict how genomic, developmental and behavioural variation influences rapid organismal adaptation. This will be beneficial to the field of evolutionary biology and also conservation biology. Further, we believe that our results will benefit several field of biological sciences (e.g., evolution, developmental biology, molecular biology, and genetics) because our project integrates across biological scales (genomics, cellular and developmental biology and behaviour). We will publish and disseminate important insights into the genes and developmental processes that generate vertebrate morphological diversity in nature and in the laboratory. Using cichlids, we will be able to connect natural variation to developmental processes that are important to the ontogenesis (period of development from embryo to adult) of all vertebrates, including humans.

How will you look to maximise the outputs of this work?

We will present our results in local and international conferences and we will publish in international open access journals (as we did and are currently doing in relation to our current regulated work) and we will share our data in online repositories to be freely used by other researchers. Conferences and publications enable us to communicate our results and experimental methods to a broad scientific community. For example, we have organised a Cichlid Genome Editing workshop bringing together a group of experts in cichlid developmental genetics. As a result of this workshop we wrote a review on the use of the method in different species. Moreover, the cichlid community in the UK is highly collaborative and meets regularly to present research updates, which increases data sharing and also increases protocol optimisation. Finally, we will contribute to public outreach activities organised by our host institution to show case our work on cichlid morphological evolution and its importance to society.

Species and numbers of animals expected to be used

- Other fish: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are using embryos, juveniles and adults of East African cichlids of the African Great Lakes due to their unique and fascinating diversity of craniofacial and body shapes, pigmentation patterns and behaviours. Although they vary in morphologies, they are genetically very similar. The combination of these features makes them a great system to

identify the genes underlying cichlid evolution. As such, there is a large scientific community that build important and useful resources (e.g. genomes and genome editing tools). They represent a model system where integrative approaches are possible (e.g. merging evolutionary biology with cell and developmental biology) thereby reducing the need to use different model system to answer related questions.

The species we proposed to use are large, robust and thrive in an aquaria environment and will withstand the proposed regulated procedures with the minimum of stress. In order to continuously minimise animal suffering we will always use up to date technology (e.g. use skin swabs instead of fin clipping when possible) and refine the housing environment. Moreover, we are up to date with current research and have refined methods of Schedule 1 killing, anaesthesia and analgesia.

Typically, what will be done to an animal used in your project?

Creation of genetic alterations: Fish eggs will be collected and placed in a petri dish with aquaria water. Then, they will be gently held and injected so as not to damage the egg. Immediately after, the eggs will be returned to aquaria. Injection of a batch of 50 eggs takes roughly 30 minutes. Injections will be performed to create genetic alterations, meaning the addition or deletion of genetic material.

Confirmation of genetic alterations: After the creation of genetic alterations, fish eggs will be grown until they reach their juvenile stage. At this point, we will confirm the presence of genetic alterations by cutting a little portion of a fin, or by taking a swab from the fish and sequence their DNA. The cichlid fish we are using are large and robust, typically about 5-10cm in length. Therefore, this procedure will be performed without anaesthesia, as this brings a higher risk of adverse effects. Melafix - a substance that fights infection and promotes fin regrowth - may be used. We will monitor for abnormal behaviour post fin clipping that might indicate pain, and if required isolate the fish and use analgesia treatments (following Named Veterinary Surgeon advice). In the past we have never witness such abnormal behaviour, so we tend to avoid analgesia as the harms of isolation would outweigh the benefits of its use. The amount of fin removed does not compromise swimming or any other aspects of normal fish physiology or behaviour, taking the sample takes less than a minute.

Repeated imaging through time: Fish showing morphological modifications (e.g., colour pattern modifications) due to the genetic alterations will be imaged throughout time, while the morphological trait (e.g., colour pattern) is developing. To improve the imaging, individuals will be anaesthetised and subjected to a substance that contracts the pigmented cells that make up the colour pattern. In these species, the development of colour patterns is highly dynamic and changes as the fish grows, therefore it is not possible to use such pigmentation markings to keep track of individuals. Thus, the fish for this experiment, may be housed singly (for a maximum period of 150 days) in order to keep track of individual identities, and while this is not the ideal situation for cichlid fishes, it will increase the value of the longitudinal imaging data while having little impact on fish growth and morphological trait formation. An added reason for the fish to be housed singly for this experiment, is that pigmentation formation is inhibited in most individuals if they are kept all together, as the presence of a dominant male will supress pigmentation development in subordinate individuals. Thus, to synchronise pigmentation pattern development between individuals (and increase replicates), each fish has to be given their own territory. We will control for signs of stress by comparing growth rates between single housed animals and animals kept in a group. Our preliminary data shows that juvenile fish kept in single housing grow at normal rates and gain colour quite rapidly.

Hormonal manipulations: Fish embryos and early juveniles will be subjected to hormonal manipulations, where certain hormonal (e.g., thyroid and sex steroids) levels will either be increased or decreased. Substances that manipulate hormonal levels will be administered by dilution in the aquaria water.

Behavioural assays: to determine the role of the genetic and morphological alteration in the reproductive and courtship behaviour of these fishes, we will perform mate-choice, male-male competition and exploratory behaviour of these species. This will allow us to measure the fitness effects of the induced genetic alterations and hormonal manipulations. We will conduct three types of behavioural assays:

For male competition, we will use long aquaria (150cm), that will be divided into five compartments. Every compartment will hold a male with different morphological attributes, the dividers will have holes where females can go through. Mating success will be measured by identifying who fathered most progeny.
For mate choice, we will have tanks with three compartments, where a female will be in the middle compartment and there will be a male on each side compartment. Mate choice will be measured as time spend with each male.
For exploratory behaviour, we will use a very large tank (150cm x 50cm x 50cm). The individual will be placed at the end of this tank, and will be allowed to roam for 15 minutes. Then its movements will be tracked using tracking software to see where and how did this individual explored it's surrounding environment.
Only fish showing no signs of suffering and distress will be used in these behavioural trials.

As these behavioural trials mimic the semi-natural conditions we rear our cichlids in we do not expect this protocol to induce any suffering, harm or distress.

What are the expected impacts and/or adverse effects for the animals during your project?

Genetic alterations and hormonal manipulations: What we propose to do to the animals will minimally impact on their health and welfare as the genetic alterations and hormonal manipulations suggested are unlikely to lead to any malformations or adverse effects during regulated stages. We will inject and treat fertilised eggs with particles that modify specific gene portions and administer substances that modify hormonal profiles. These modifications might also result in harmful embryonic growth malformations (e.g. fish embryos that do not grow, embryos that lack organs, hatchlings that swim and behave abnormally, etc.). If the latter occurs affected animals will be humanely killed as soon as the malformation is detected before they hit the independent feeding stage.

Repeated imaging: Fish showing colour modifications will be imaged throughout time, while the colour pattern is developing. To improve the imaging, individuals will be anaesthetised and subjected to a substance that contracts the pigmented cells that make up the colour pattern. The repeated exposure to this substance and to anaesthetic can lead to a 5% rate of non-recovery from the anaesthetic. Imaging will first be performed every five days to limit exposure to the anaesthetic and to the substance that causes pigment cells to contract. We will only shorten the interval between imaging sessions if the morphological trait develops too fast for differences to be detected with larger intervals. Animals that are not fully recovered at the end of this procedure will be killed humanely. Further, the fish for this experiment, may be housed singly in order to keep track of individual identities, and while this is not the ideal situation for social cichlid fishes, this will increase the value of the longitudinal imaging data (e.g., we will be able to follow individual cells) while having little impact on fish growth and morphological trait formation. The

pigmentation traits we are interested in are related to dominance and their development/appearance is inhibited by conspecifics, only showing up in the most dominant fish. Therefore to synchronise trait development across all individuals we need to isolate them so that each has their own territory.

Behavioural trials: We expect no adverse effects. This protocol is considered to be regulated because we will be using genetically altered animals and animals that underwent hormonal manipulations. Only health fish showing no signs of suffering and distress will be used in these behavioural trials. As these behavioural trials mimic the semi-natural conditions we rear our cichlids in we do not expect this protocol to induce any suffering, harm or distress. Nonetheless, we will closely monitor the fish to detect any signs of suffering and stress during the procedure and in 24 hours following the procedure.
East African cichlids are large and robust species which will withstand well our proposed procedures. After any procedure, animals will be monitored for an adequate amount of time to ensure recovery. Animals are expected to remain healthy and normal and, should this not be the case, they will be humanely killed immediately to prevent any pain, suffering, or distress.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild Severity - 100%

What will happen to animals at the end of this project?

- Kept alive
- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Our research focuses on understanding how vertebrate diversity is generated at the genetic and developmental level. Correct development depends on cell and tissue interactions present in the embryo. Therefore, the study of development cannot be replaced by in vitro or ex vivo models, since the interaction with the live and intact tissue is needed.

The genetically altered lines generated and tissues collected will be made openly available and shared with the scientific community (with necessary permissions) which will replace/reduce animal use in the collaborator’s laboratory.

Which non-animal alternatives did you consider for use in this project?

We have considered mathematical models and cell cultures.

Why were they not suitable?

At the moment there are no alternatives that faithfully replace animal development from a single cell to its adult form. To fully understand how genetic mutations and hormones impact animal development, we need to observe how animals develop from embryos to adults in a natural context. Cell cultures and mathematical models are not able to recreate complex interactions that occur at the organismal level between cells and tissues. Furthermore, part of our project also focuses on the impact of genetic and developmental changes in behavioural profiles of these fishes. This integrative approach would be impossible without using animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We plan to generate up to 15 lines of genetically altered animals. From past experience we estimate that for their generation and breeding we will need approximately 1500 animals. This is roughly 50 injected eggs per gene that will grow to sexual maturity and then crossed to generate a stock of 50 F1s per line.

We will then repeatedly image the embryonic development of such lines. We estimate (based on previous studies) that we will need at least 12 individuals (6 females and 6 males) per group/treatment. Here we will use these lines to:

Compare the development of wild type and genetically altered individuals. This will require 12 individual per group (wild type and GA) and per line (15 lines), thus we will require a maximum of 360 individuals.

Compare the development of wild type and GA animals reared in normal conditions with animals with hormonal levels manipulated. This will require 12 individuals per group and we plan to use a maximum of 5 GA lines. The studied groups will include GA and wild type animals reared under normal condition, treated with hormones and the vehicle substance, and vehicle substance only. We plan to use four types of hormonal manipulations (enhancement and inhibition of thyroid and sex steroid hormones). This will require a maximum of 1440 animals.

Finally, we plan to determine the impact of genetic alterations and hormonal manipulations in the reproductive and exploratory behaviour of these fishes. This will require 15 individuals per group and we plan to use a maximum of 5 GA lines. The studied groups will include GA and wild type animals reared under normal condition, treated with hormones and the vehicle substance, and vehicle substance only. We plan to use four types of hormonal manipulations (enhancement and inhibition of thyroid and sex steroid hormones). This will require a maximum of 1800 animals.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Genetic alterations will be performed on a carefully chosen set of genes. At the beginning of this research programme, we used computational methods and available data to confirm that the candidate genes are associated with morphological variation. For example: 1) we will perform comparative transcriptomics experiments to analyse gene expression profiles for the tissues of interest between species, and; 2) we will perform hybrid crosses between species and sequence the genome of hybrids to perform genomic mapping of genes underlying morphological variation. These experiments will yield very specific candidate genes that are expressed in the tissues of interest and that have mutations causally associated with variation in the trait of interest. As such, we will only study the gene function of highly relevant candidate genes.

During our previous licensed work, we have gained experience that allow us to determine the ideal numbers of animals for each of the protocols in this licence. More specifically, we published work on genome editing in cichlids and on the repeated imaging of genetically altered animals and as such could determine the ideal number of animals required to generate interpretable results. We have also conducted a few pilot experiments on hormonal manipulations to determine dosage and numbers required to measure morphological effects. Finally, we have conducted pilot trials and discussed with colleagues in cichlid behavioural ecology who advised on the numbers required to conduct pilot experiments for each of the behavioural trials.

We will strive to use the optimum number of animals and throughout the duration of the project we will continuously consider if the benefits of the number of animals used and procedures performed outweigh the potential harm. We will plan, conduct and report our experiments according to the PREPARE and ARRIVE guidelines and use the NC3Rs Experimental Design Assistant (https://www.nc3rs.org.uk/our-portfolio/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?

Efficient breeding: For the generation and breeding of cichlid genetically altered strains, only individuals with confirmed gene modifications will be used. Gene modifications are confirmed by collecting tissue biopsies (e.g. by removing a very small piece of fin or collecting skin swabs), which are then used for genetic tests.
Pilot studies: We commit to perform pilot studies for protocol we have less experience with, namely hormonal manipulations and behavioural assay. We will consult the NC3Rs Experimental Design Assistant (see above) to help design and conduct the pilot studies (e.g., https://www.nc3rs.org.uk/3rsresources/conducting-pilot-study).
Sharing tissues and data: We will share tissues from the fish we cull with collaborators within the University. For every fish culled in our facility we always enquire if tissues are needed, so that animal use is optimised. We also publish our results in open access journals and upload all raw data to data repositories and our group's GitHub page.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the

mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 as a model species East African cichlid fishes. We will generate genetically altered lines, conduct hormonal manipulations, image trait development and conduct behavioural trials. All our proposed procedures are mild.

The species group we study are evolutionary important and there is a large scientific community that build important and useful resources (e.g. genomic and molecular tools). They represent a model system where integrative approaches are possible (e.g. merging evolutionary biology with cell and developmental biology) thereby reducing the need to use different model systems to answer related questions.

The species we proposed to use are large, robust and thrive in an aquaria environment and, we know from our past and current experience, that they will withstand the mild regulated procedures with the minimum of stress. In order to continuously minimise animal suffering we will always use up to date technology (e.g. using skin swabs instead of fin clipping when possible) and refine the housing environment. Moreover, we are up to date with current research and refining methods of Schedule 1 killing, anaesthesia and analgesia.

Why can’t you use animals that are less sentient?

A major component of our research focuses on understanding how vertebrate diversity is generated at the genetic and developmental level. We have a particular interest in the development of tissues derived from neural crest cells, namely colour patterns and craniofacial skeleton, which only exist in the vertebrate group. Our questions are thus centred on vertebrate biology and evolution, as such we cannot use less sentient species. Correct vertebrate development depends on cell and tissue interactions present in the embryo and juvenile fish. To properly characterise such processes we need to image the same individuals through time - from embryo to juvenile and adult periods - to be able to document how, for example, pigmentation and craniofacial shapes develop. Thus, we cannot solely use immature life stages or animals after they have been terminally anaesthetised.

However, we are committed to reduce animal numbers, and will whenever is possible use imaging data and tissues obtained from immature life stages (before independent feeding stage) and terminally anaesthetised animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will incorporate new recommendations on animal rearing and welfare as we have done up to now. Cichlid housing will be continuously improved with rocks, vegetation and hiding places characteristic of their natural environment. This will be achieved by introducing artificial plants, stones, clay pots, and plastic tunnels in the aquaria. Group housing decreases stress and aggression levels in fish. We will maintain adult fish in high-density groups to reduce aggression, thus avoid unnecessary stress or injuries from chasing or fighting. Researchers and animal technicians will pay close attention to the fish in every

tank, in order to control stress and aggression levels. If required, fish will be temporarily separated and groups redesigned to avoid further injury and aggression. In case of signs of aggression (e.g., bitten fins or after fin clipping for confirmation of genetic alteration, melafix (substance that promotes fin regeneration and growth) can be added to the aquaria water to promote regrowth of affected fin areas. We will continuously run trials on optimising feeding regimes and control water quality to ensure optimal husbandry. We perform fish health and welfare checks twice a day.

We commit to continuously work on our protocol refinement, for example, under our current project licence, we have: 1) optimised cichlid genome editing protocols ; 2) refined a protocol of repeated anaesthesia and longitudinal imaging of cichlid fish, which we will use in this project; 3) refined housing environment and husbandry techniques (e.g., we maintain and breed cichlid species that rarely do so inn a laboratory setting); 4) applied skin swabs as a DNA sampling method for larger specimens; 5) shared such protocols with the cichlid community. Further, using immature life stages (embryonic stages) we have conducted preliminary hormonal manipulation studies which informed the viable concentration ranges to use in our experiments. These concentrations ranges result in viable late embryos, as such we think it is safe to apply these ranges past the independent feeding stage.

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 available at www.nc3rs.org.uk. Experiments will be planned, documented, and reported according to the PREPARE and ARRIVE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We stay up-to-date with new information and new resources as they become available. We are signatories of the NC3Rs (National Centre for the Replacement, Refinement, and Reduction of Animals in Research) newsletter and follow their website (www.nc3rs.org.uk). Moreover, named persons in our establishment (i.e. the Named Animal Care and Welfare Officer - NACWO, Named Veterinary Surgeon - NVS, and Named Information Officer - NIO) inform us of relevant new information and resources. My research group is in constant contact with our NACWO, NVS and animal care staff, to discuss and exchange improvements in animal rearing, wellbeing and implementation of new resources.